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Persistent harmful scenarios associated with disposal of radioactive waste, high-background radiation areas and severe nuclear accidents are of great concern regarding consequences to both human health and the environment. Of particular concern is the extracellular DNA in aquatic environments contaminated by radiological substances. Strand breaks induced by radiation promote decrease in the transformation efficiency for extracellular DNA. The focus of this study is the quantification of DNA damage following long-term exposure (over one year) to low doses of natural uranium (an alpha particle emitter) to simulate natural conditions, since nothing is known about alpha radiation induced damage to extracellular DNA. A high-resolution Atomic Force Microscope was used to evaluate DNA fragments. Double-stranded plasmid pBS as a model for extracellular DNA was exposed to different amounts of natural uranium. It was demonstrated that low concentrations of U in water (50 to 150 ppm) produce appreciable numbers of double strand breaks, scaling with the square of the average doses. The importance of these findings for environment monitoring of radiological pollution is addressed.

1. Introduction

1.1 Uranium in the environment

Worldwide concern regarding the environmental impact and health risks as a consequence of exposure to ionizing radiation, naturally occurring or produced by anthropogenic activities, is well-known. Uranium is of particular concern, firstly because of its ubiquity and secondly because of its use as power generating fuel. In fact, mining, processing and use of uranium are big issues regarding possible impacts to the environment and on local populations. The risk of contamination of food (fruit, vegetables, meat, etc.) and drinking water must be also considered. Natural uranium, which has a $^{235}$U content of 0.7%, is a radioactive material that emits ionizing alpha, beta and gamma radiation. The effects of exposure, even to natural uranium, are both radiological and chemical (heavy metal toxicity) in nature. Besides uranium production facilities, other contributors of possible radioactive contaminations are the natural radionuclides concentrated by non-nuclear technologies as coal burning, fertilizing with phosphate fertilizers (that contain uranium), leaching from natural deposits, release in mill tailings and basic chemistry. Those are the so-called technologically enhanced, natural occurring radioactive materials (TENORM). Concern regarding TENORM arises from the fact that these materials are anthropogenic in nature. Actually, some materials with a high $^{234}$U/$^{226}$Ra ratio (as e.g. phosphate fertilizers and sodium
tripoliphosphate) are identified as contributors to $^{238}$U contamination.²

1.2 Uranium in water – effects on humans

The general population is exposed chronically to various levels of uranium by inhalation, dietary habits and drinking water.³ It has been estimated that the daily uranium intake for residents in São Paulo City (Brazil) is 1.0 $\mu$g,³ while for Canadians it is approximately 2.6 $\mu$g,⁴ with food accounting for 77% and water for the remaining (approximately 0.8 $\mu$g).

Intake of uranium through drinking-water is naturally low. There are reports, however, that the amount of uranium ingested via water could be much higher in areas where uranium concentration in drinking water exceeds 2 $\mu$g l⁻¹.⁵

Although little information is available on the chronic health effects of exposure to environmental uranium in humans, it is known that nephritis is the primary chemically induced effect. Uranyl nitrate was cytotoxic and genotoxic in Chinese hamster ovary cells at concentrations ranging from 0.01 to 0.3 mmol per litre. Chromosomal aberrations, on the other hand, were induced in male mice germ cells exposed to enriched uranyl fluoride; however, these aberrations may have been produced by the radioactivity of the test compound.⁶

1.3 Uranium in water – burden on biotic and abiotic materials

Unfortunately, contamination of water bodies has been occurring all over the world. For instance, just to mention a recent case, the Bega canal is one of many heavily polluted canals in Vojvodina (the northern province of Serbia and Montenegro). It was found that the Bega sediment is contaminated with $^{238}$U and $^{137}$Cs, although no traces of contamination by nuclear power plants in the region were found. However, the presence of TENORM contamination was detected.² Thus, monitoring of radionuclides in water (uranium, in particular) should also be high in the environmental protection agenda.

The least well-studied harm to the environment caused by uranium in water is on the biota. In fact, changes occurring in the environment, e.g. by radiological pollution, constitute significant burdens to biota.⁷ In this sense, biological monitoring could work as an early warning system to detect changes driven by contaminants.

As pointed out elsewhere,⁸ however, it is also important to study alterations in physical structures of abiotic materials (such as extracellular DNA) because ecosystems consist of both biotic and abiotic components. Some bacteria can develop competence for extracellular DNA uptake and express the foreign genetic material,⁹,¹⁰ indicating that extracellular DNA serves as a material determining bacterial characteristics and their genetic functions through natural genetic transformation.

In this regard, Ishii and collaborators⁸ studied the impact of gamma irradiation on the transformation efficiency of extracellular DNA by using a plasmid as a model for extracellular DNA. Transformation efficiency is a measure of a cell’s ability to undergo genetic transformation, defined as the number of transformed cells (transformants) generated by a unit weight of extracellular DNA. Competent cells of E. coli DH5α were used to examine the plasmids irradiated at a reasonably high gamma dose of 200 Gy, far from natural conditions. Strand breaks were identified through agarose gel electrophoresis. It was concluded that the transformation efficiency decreased with the increase in radiation dose, induced by single and double strand breaks, thus suggesting an inability of microorganisms to acquire new characteristics, which should be normally acquired.

A number of studies have been carried out on the effects of gamma irradiation on extracellular DNA,¹¹–¹³ but none on the effects of alpha particle radiation, the highest LET (Linear Energy Transfer) radiation decaying from natural uranium, a persistent radiological contaminant of water at various levels. We note, in this regard, that extracellular DNA is a component of aquatic ecosystems because it is ubiquitous in freshwater and marine environments at concentrations from 0.5 to 88 mg l⁻¹.¹⁴,¹⁵

Although a relatively long-lived radionuclide, natural uranium emits 4.2 MeV alpha particles which have the highest LET of environmentally occurring radiation. However, as recently demonstrated, radiation damage occurs not only by the high-energy incident particles of this radiation, but also by the low-energy secondary electrons that they produce. These studies have demonstrated that a large fraction of these electrons are generated by a relatively unusual auto-ionization process known as intermolecular Coulombic decay.¹⁶,¹⁷ Roughly, 5 $\times$ 10⁴ secondary electrons are produced per 1 MeV primary energy of photons.

In the present work, therefore, realistic environmental conditions are simulated for aquatic environments contaminated by natural uranium, plus a plasmid as a model for the extracellular DNA content in water. The focus of the study is the identification and quantification of DNA radiation damage following long-term (over one year) exposure to uranium, an alpha-particle emitter, using high-resolution Atomic Force Microscopy to evaluate DNA fragment size profiles. DNA fragments are produced by multiple double strand breaks (DSBs), and these strand breaks are closely related to the decrease in the transformation efficiency for extracellular plasmid DNA, as suggested elsewhere.⁸ It is our hope to provide guidelines for the planning and implementation of biological monitoring, particularly for an earlier detection of contaminants in water bodies.

2. Materials and methods

2.1 DNA sample preparation

Double-stranded pBS plasmid DNA (this is the plasmid Bluescript II KS⁺) was used, prepared at a concentration of 1.0 mg ml⁻¹ in TE buffer (10 mM Tris–Cl, pH 7.5 and 1 mM EDTA). This plasmid is 2961 base pairs (bp) in length (~940 nm, assuming 3.4 nm per 10.5 bp for B-type DNA⁹ with a width of 1.9 nm). For irradiation and AFM imaging, the naked DNA plasmid was diluted at 5.0 $\mu$g ml⁻¹ in 10 mM Hepes and then divided into aliquots in 0.5 ml plastic microcentrifuge tubes, each containing 100 $\mu$l of the DNA solution. No additional free radical scavenger was added to the Hepes buffer. The extraction and purification of DNA were done using the “Invisorb Plasmid Kit”, according to the manufacturer’s specifications. Linearization was performed with the enzyme HindIII, also following the manufacturer’s specifications. Finally, in order to prevent rejoining, the linearized DNA was dephosphorylated.
with the aid of the enzyme “Calf Intestine Alkaline Phosphatase” (CIAP). All samples were exposed to air during their handling.

2.2 Extracellular plasmid DNA irradiation

Seven samples of PBS DNA plasmid, all of them with the same concentration of 5 ng μl⁻¹, were diluted in 5 ml of distilled water inside 7 microtubes with a capacity of 10 ml. Uranium (with natural isotopic composition), in the form of uranyl nitrate at a concentration of 1.0 g l⁻¹ (of uranium) was added to 6 microtubes in aliquots of 50 μg (2 microtubes), 100 μg (2 microtubes) and 150 μg (2 microtubes). One uranium free microtube worked as control. This set of 7 microtubes was accommodated over a laboratory bench at room temperature, remaining untouched for a period of 12 months and then prepared for DNA imaging with AFM.

2.3 DNA imaging with AFM – distribution of fragment length

The microscopes used in this study are the AutoProbe CP (ThermoMicroscopes) and the Agilent-5500. For DNA imaging, the AFM was operated in air and non-tapping mode. Conical silicon tips were used with a typical curvature radius of 10 nm and aspect ratio of 3 : 1, mounted on cantilevers with resonance frequency around 80 kHz and a spring constant in the range of 1.8 to 3.2 N m⁻¹. Sample preparation for AFM imaging consisted of the deposition of 1.0 μl of aqueous DNA solution on an atomically flat mica surface, followed by a gentle rinse using a few drops of distilled water. 1.0 mM MgCl₂ was deposited on the mica substrate to assure the adhesion of DNA molecules. Before placing the DNA sample on the mica foil, MgCl₂ excess was removed by washing with distilled water. The DNA sample on the mica was then dried in a gentle flow of nitrogen gas for 5 min and DNA fragment-length measurements were performed with the Agilent-5500 software, allowing segmented measurements of a curved fragment. The length of each segment was measured and accumulated to yield the length of the entire curved fragment. The resolution of the AFM in these measurements was in the range of 2 to 6 nm (6 to 17 bp). Five aliquots of 1.0 μl aqueous DNA solution were retrieved from each microtube. Ten 2 μm × 2 μm images were taken and scanned from each 1.0 μl aliquot, totaling 50 images for each microtube. The number of measured DNA fragments per image ranged from 150 to 300, providing a huge number of fragments for each uranium dose. An average of all data was calculated, and dispersions equal or smaller than 10% were obtained. The number of DNA fragments as a function of their length was normalized to the total number of scanned fragments.

3. Results and discussion

Results of DNA plasmids exposed for a period of 12 months to radiation decaying from natural uranium are shown in Fig. 1. Two columns are displayed in this figure, one with the AFM images and another showing the normalized number of plasmid fragments as a function of their length.

3.1 Fragment distributions

Although amounts of natural uranium were quite low (50 μg, 100 μg and 150 μg), so as to simulate mild environmental contamination, we observed patterns of DNA fragment distributions scaling consistently with their respective doses. Patterns, distributions, etc., are classified in Quantum Mechanics terminology as ‘continuous’ (smooth) or ‘discrete’ (in pieces). Interestingly, the fragment distributions revealed discrete-like patterns for the three uranium doses. This is at variance with higher dose (100 Gy) results obtained from irradiation with 6 MeV electron beams,\(^\text{20}\) which produced substantial number of fragments of all lengths (Fig. 2), a likely consequence of the multi-hit character of radiation interaction at these dose and energy conditions (see discussion in Section 3.3 below).

Considering that 1.0 mg of uranium emits 12 alpha particles per second (calculation details in ref. 21), then 150 μg would emit about 5.7 × 10⁷ alpha particles per year inside a microtube with 9 × 10⁶ plasmids, approximately. Therefore, it would be an average of 6 alpha particles (energy of 4.2 MeV each) available per 1000 plasmids. Actually, 40% of the DNA plasmids were shattered by radiation emitted by 150 μg of uranium – see Fig. 1D (about 60% of the plasmids remained intact after one year of exposure). This is evidence that the plasmids were not shattered only by direct interactions with alpha particles, which makes the effect of secondary electrons (see Introduction) of importance too.

3.2 Radiation interaction and average dose estimates

Alpha particles (heavy charged particles) crossing matter interact mostly by inelastic collisions with atomic electrons. Their energy is lost by atomic excitation and ionization, and a small fraction is lost by production of low-energy secondary electrons (see Introduction). Thus, the velocity of an alpha particle gradually slows down after interaction with electrons, since in each collision the maximum energy transferred (\(E_{\text{max}}\)) is \(4ε_a m_α/m_0\), where \(ε_a\) is the alpha particle energy, and \(m_α\) and \(m_0\) are the masses of the alpha and electron, respectively. Since natural uranium emits 4.2 MeV alpha particles we have that \(E_{\text{max}} \approx 2.3\) KeV; thus, an alpha particle stops only after undergoing approximately 1800 inelastic collisions. Therefore, trajectories of alpha particles in the matter are nearly straight lines because \(m_α \gg m_0\). In this sense, moderation of DNA exposure to radiation by solutes in natural water turns out to be quite small, particularly if we also take into account the proximity of the plasmid DNA with the uranyl molecules in the water.

As pointed out elsewhere,\(^\text{8}\) doses equal to, or higher than 100 Gy are very far from natural conditions, occurring only in accidents such as in the Chernobyl nuclear power plant (1986) – absorbed gamma doses measured in pine tree needles found close to the damaged reactor were higher than 100 Gy.\(^\text{23}\)

In the present experiment, known amounts of natural uranium were dissolved in water containing known masses of DNA plasmids. The classical definition of absorbed dose, as used in medical therapy and accidental exposures, is

\[
D = \frac{de}{dm_\alpha}
\]
where, in our case, $\varepsilon$ is the energy transferred to the water plus plasmid solution, and $m_p$ is the solution mass. This is a small amount of energy deposited in a small mass of solution.

However, it suffices, for the purposes of this work, estimates of average doses only. In fact, the adoption of average doses is very appropriate for radiation protection, which could be defined as\(^\text{21,23,24}\)

$$D_{av} = N_a \left[ \frac{\varepsilon_a}{m_p} \right]$$  \hspace{1cm} (2)

**Fig. 1** AFM 2 $\mu$m $\times$ 2 $\mu$m image sequences and the corresponding distributions of fragments from pBS plasmids (normalized histograms) exposed to different amounts of uranium (details in the text): (A) 0 $\mu$g, control sample, (B) 50 $\mu$g, (C) 100 $\mu$g, and (D) 150 $\mu$g.
and N mentions (those /C24 Atomic Force Microscopy carried out at this laboratory. A degree of accuracy is possible only with the high-resolution measurements from 50 AFM images. We can therefore reliably histograms shown in Fig. 1B–D represent the averaged providing 50 images for each microtube. Thus, each of the each U content (50, 100 and 150 again (see details in Section 2.3) that 2 microtubes were used for 20% and 38% (Fig. 1 and 3). It is important to emphasize adversely, corresponding to percentages of shattered fragments equal to 3.5%, 20% and 38% (Fig. 1 and 3). It is important to emphasize again (see details in Section 2.3) that 2 microtubes were used for each U content (50, 100 and 150 µg), with AFM scanning providing 50 images for each microtube. Thus, each of the histograms shown in Fig. 1B–D represent the averaged measurements from 50 AFM images. We can therefore reliably

Fig. 2 Distribution of fragments size (L) of plasmids irradiated (a) with an electron dose of 100 Gy (adapted from ref. 14), and (b) with radiation decaying from natural uranium (this work).

for irradiation from internally retained radioactive materials; \( e_a \approx 4.2 \text{ MeV} \) is the energy of an alpha particle emitted by \(^{238}\text{U}\) and \( N_a \), the number of alpha particles emitted during a time exposure \( \Delta t \), is given by\(^{21} \)

\[
N_a = 0.0123 m_U (\mu g) \Delta t (s) \tag{3}
\]

\( m_U \) is the total natural uranium mass dissolved by water in microtubes containing DNA plasmids (see Materials and Methods). We have used 50, 100 and 150 µg of uranium, which correspond to concentrations of 10, 20 and 30 ppm (U) respectively, in the distilled water inside microtubes. The corresponding average dose estimates after one year of exposure were of the order of cent-Grays (cGy), ranging from 5 to 15 cGy, approximately.

3.3 Radiological effects on the DNA plasmid

We used plasmids after their linearization, which makes AFM imaging more easy and precise. Thus, the total number of fragments measured for each dose equals the total number of double strand breaks (DSBs) induced in the DNA plasmids, since only a DSB could scission the molecule. We observe in passing that single strand break (SSBs) damage to the deoxyribose–phosphate backbone in two nearby locations can lead to scission of the molecule, a damage referred to as a DSB.\(^{24} \) The consistency of our results is also revealed by the fact that the majority of fragment lengths observed in the present work (Fig. 1) can be arranged into pairs, summing up \( \sim 940 \text{ nm} \) (the total plasmid length). This degree of accuracy is possible only with the high-resolution Atomic Force Microscopy carried out at this laboratory.

We observe in Fig. 1B–D that the percentages of intact fragments (those \( \sim 940 \text{ nm} \) long) are 96.5%, 80% and 62%, respectively, corresponding to percentages of shattered fragments equal to 3.5%, 20% and 38% (Fig. 1 and 3). It is important to emphasize again (see details in Section 2.3) that 2 microtubes were used for each U content (50, 100 and 150 µg), with AFM scanning providing 50 images for each microtube. Thus, each of the histograms shown in Fig. 1B–D represent the averaged measurements from 50 AFM images. We can therefore reliably

assume that the percentages 3.5%, 20% and 38% are DSBs probabilities \(( F_{DSB} )\), within experimental uncertainties smaller than 10% (not shown in Fig. 3). A best fitting procedure to these data provided the function (solid curve in Fig. 3)

\[
F_{DSB} = 0.18 m_U^2 (m_U = 0, 50, 100 \text{ and } 150 \mu g). \tag{4}
\]

The zero Uranium point \(( m_U = 0 \mu g, F_{DSB} = 0\% )\), which is the 4th point used in the analysis, is unquestionably a very strong fitting constraint, since it reduces to 2 the number of fitting parameters, that is, \( F_{DSB} = am_U^b \). The obtained chi-square per degree of freedom was very close to one. Attempts to fit straight lines \(( b = 1 )\) or higher order polynomials \(( b > 2 )\) resulted in chi-squares much larger than one.

It is straightforward to show that doses \(( D )\) are proportional to \( m_U \), implying \( F_{DSB} \) proportional to \( D^2 \) (see eqn (4)), that is,

\[
F_{DSB} = K_{DSB} D^2, \tag{5}
\]

where \( K_{DSB} \) is an ad-hoc proportionality constant.

This finding deduced from our experimental data (Fig. 1), plus the assertion that the total number of DNA plasmids fragments equals the total number of DSBs (Fig. 3), is consistent with radiobiological concepts because,\(^{24} \) (a) the yield (or probability) of SSB lesions \( y_{SSB} \) is proportional to the absorbed dose, that is

\[
y_{SSB} = K_{SSB} D, \tag{6}
\]

where \( K_{SSB} \) is another proportionality constant, and (b), DSBs result from cooperative interaction of two neighboring SSBs. In this sense, the yield (or probability) of DSBs, \( y_{DSB} \), could be written as

\[
y_{DSB} = K_C y_{SSB} y_{SSB} = K_C K_{SSB} D^2 \equiv K_{DSB} D^2, \tag{7}
\]

where \( K_C \) is some sort of coupling factor, here phenomenologically introduced.

It is important to point out, for instance, that from the valid theory of dual radiation action, low LET radiation effects in cells are described by the square of the dose, suggesting an interaction of two consecutive single events.\(^{28} \)
3.4 Ecotoxicology aspects and significance of results

This work primarily focused on the identification and quantification of radiation damage in extracellular DNA following long-term exposure to uranium – a uranium polluted scenario was simulated. The main motivation resided in the fact that extracellular DNA is an abiotic substance essential as genetic material for all organisms. In this sense, toxicological issues of this study are not related to radiation burden in humans interacting with aquatic environments contaminated by natural uranium.

Uranium concentrations studied in this work are high in relation to water quality standards (1–2 μg l⁻¹), but not in relation to e.g. the lowest-observed-adverse-effect level (LOAEL). As discussed in the Introduction, however, the presence of TENORM in the environment increases radiological pollution, particularly in water where extracellular DNA is found.

In this regard, the first time observation of a quadratic relationship between radiation damage of extracellular DNA and dose (Fig. 3) is of great importance for the environmental monitoring of radionuclide burden to abiotic and biotic materials. For instance, if the concentration of uranium is determined in a water body by means of routine monitoring, the level of radiation damage to the extracellular DNA would be inferred from interpolation or extrapolation in the curve exhibited in Fig. 3.

4. Conclusions

The conclusions of the present work lead to the following conclusions:

(1) Doses as low as 15 cGy from natural uranium produce appreciable amounts of DSBs, which affects the transformation efficiency of extracellular DNA.

(2) The radiation emitted from natural uranium yields DSBs scaling with the square of the doses, quite consistently vis-à-vis radiobiological concepts. In this sense, our experimental results do not contradict the assumptions expressed by eqn (2) and (4).

(3) The qualitative conclusion that transformation efficiency decreases with the increase in radiation dose, as stated elsewhere, could now be quantitatively rephrased as transformation efficiency decreases directly proportional to the square of the radiation dose (Fig. 3).

It is worthwhile stressing that we have simulated two quite important and realistic conditions associated with contamination of water bodies by natural uranium: (i) low doses and (ii) very long exposure. Instead of agarose gel electrophoresis, on the basis of water bodies by natural uranium: (i) low doses and (ii) very long exposure. Instead of agarose gel electrophoresis, on the basis of water bodies by natural uranium: (i) low doses and (ii) very long exposure.

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