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Microarray technology for the study of DNA damage by low-energy electrons

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Abstract. The damage induced to a model DNA (dT_{25}) immobilized on a gold surface by the interaction of low-energy (1 eV) electrons was studied by means of microarray technology. High quality single-stranded DNA arrays were hybridized with a dye-marked complementary strand after irradiation with electrons and the normalized fluorescence data were used to quantify the DNA damage. The data clearly show the sensitivity of the method. A significant loss of genetic information was already observed at dose as low as few hundred of electrons per immobilized oligonucleotide. The results imply that single stranded DNA and RNA are appreciably more sensitive to radiation and the attack of secondary electrons during replication, transcription or translation stages than the current radiation damage models envisage.

PACS. 34.80.Ht Dissociation and dissociative attachment by electron impact – 34.80.Lx Electron-ion recombination and electron attachment – 87.14.Gg DNA, RNA

1 Introduction

Experimental evidence is emerging [1–3] for efficient damage of DNA by low energy electrons (below the ionization energy) inducing among others single and double strand breaks [1]. At very low energies (<3 eV) only single strand breaks occur [3]. Dissociative-Electron-Attachment (DEA) is considered as the initial process involving molecular shape-resonances situated at the nucleotide bases and the sugar unit [3]. Low energy electrons are present in exceeding amounts in a living cell as secondary species following the interaction with high energy quanta (photons, particles) [4]. The understanding of these processes on a molecular scale is important not only from the point of view of fundamental science, but also for estimating the effects when human beings are continuously exposed to very low doses of radiation, one of the major problems in public health today. The aim of this work is the development and application of microarray technology to address these questions. In an initial study [5] we were concerned meanly with the feasibility of such an approach, whereby relative large electron doses were applied. Here we present more detailed and quantitative study at few orders of magnitude lower electron doses.

So far, the interaction of slow electrons with DNA has been assessed on the basis of electron induced fragmentation reactions of volatile DNA components (bases and sugars) by mass spectroscopy (ionic and neutral fragments) [2,6–10]. In only one case a different method (electrophoreses) has been applied to reveal the formation of single-strand-breaks (ssb) and double-strand-breaks (dsb) in plasmid DNA at electron energies as low as 3 eV [1]. The advantage of the whole range of high throughput methods recently developed in biochemistry and molecular genetics has so far not been explored. Methods based, for example, on a microarray technology [11] offer an extraordinary potential to assess the reactivity of a large number of DNA sequences towards low energy electrons very efficiently. As many as several hundred thousand DNA probes of known sequence, precisely positioned on a DNA chip of about 1 cm^2 area are commercially available nowadays. The methods like hybridization (the process of formation of a DNA double strand from two complementary single strands) in combination with measuring fluorescence from dyes attached to DNA permit addressing the characteristics of DNA probes in a highly parallel, rapid fashion. Therefore, the application of such high throughput techniques in the field of electron-DNA interaction might lead to a large data base with detailed information on the sequence dependence of the DNA-electron interaction.

2 Experimental

The single stranded DNA oligonucleotides were obtained from Thermo Electron (Ulm, Germany). They were purified by HPLC, verified in the molecular weight by mass

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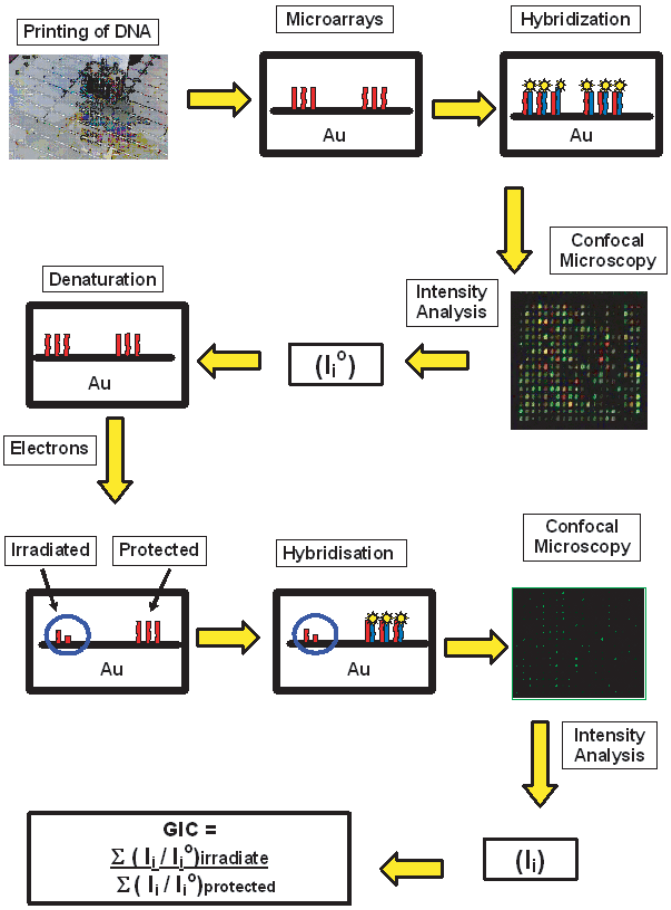

Fig. 1. Schematics of the method and evaluation procedure used to assess the low-energy electron damage to single stranded DNA immobilized on a gold surface.

spectrometry (MALDI), and had the following structure: 5′-SH-$(CH_2)_6$-dT_{25}-3′ (T = thymine). The SH-$(CH_2)_6$ link of the oligonucleotides served to immobilise the DNA strands in a defined fashion on a gold surface by the strong Au-S bond [12]. The complementary ologonucleotides marked with Cy5 dye (5′-Cy5-A_{25}-3′) were used for the hybridization. Gold chips were obtained from Arrandee (Werther, Germany). They consisted of glass pieces precisely machined ($12 \times 12 \times 1$ mm) and covered with a 2 nm layer of Cr on top of which 200 nm Au was deposited. The chips were cleaned with hot nitric acid, followed by washing with ultra pure water (Merck, HPLC grade) and drying with nitrogen gas.

The experimental method is shown schematically in Figure 1. The dT_{25} oligonucleotides were dissolved in water (100 μM), diluted in 3 × SSC (sodiumcitrate-sodiumchloride) buffer (Serva, Heildeberg, Germany) to a final concentration of 20 μM, aliquoted and stored until use at –20 °C. Aliquots were thaw, mixed and 10 μl of the solution was transferred to a microtiterplate (Genetix, New Milton, UK, X6004). The DNA solution was deposited at a relative humidity of 70% onto the Au chips using a modified Genetix QArray Microarrayer and Telechem (Sunnyvale, CA, USA) SMP4 split pin. The Au chips were fixed in the Microarrayer by means of 1×3 inch aluminium adopters into which the chips were embedded with the gold surface being planar to within 20 μm with the upper face of the adopters. The split pin was inked in the DNA solution for 3 s, and after 25 pre-spots (on a separate glass substrate) 68 spots were deposited in a soft-touch mode onto the gold substrate with the pin placed at each spot position for 100 ms. This was followed by washing of the pin twice with distilled water and drying with oil-free pressured air before further DNA transfer.

Five different sub arrays were deposited: one (8×8) array in the centre of the Au substrate (exposed in the subsequent experiments to the electron beam) and the three (4×4) and one (5×5) control arrays at the corners on the gold chip used for normalization and verification of the evaluation. Upon the completion of the printing the Au chips remained at the elevated humidity of 70% for further three hours.

After the printing of the arrays, the chips were hybridized with the complementary DNA strand (2.5 μM Cy5-dA25 in 3 × SSC for 2 h), washed two times with 3 × SSC (5 min under shaking), and further washed with copious amounts of ultra pure water (HPLC grade water, Merck) and finally dried with nitrogen gas. The samples were then scanned using an Affymetrix 418 Array Scanner (635 nm excitation; 100% laser power; 70 PTM Gain setting) and the above described aluminium adopters. The fluorescence images were analyzed with the ScanAlyze software (Stanford University, USA; `http://rana.lbl.gov/EisenSoftwareSource.htm`). Area intensity values were obtained for each spot of about 120 μm diameter at 10×10 μm pixel size. The intensities of the spots before bombardment with electrons (I_i°) were used for normalization of the corresponding spot intensities after interaction with electrons (I_i).

Following the scanning, the immobilized DNA oligonucleotides were denaturized by immersing Au chips for 30 s into 95 °C water. Repeated denaturation-hybridization cycles resulted only in small but comparable loss of the fluorescent intensity (Fig. 2). After denaturation the Au chips were immediately transferred into a UHV chamber equipped with a rotateable sample holder, Helmholtz coils and an electron gun. The small chamber was rapidly evacuated with a sorption pump, permitting opening of a straight-through valve to a turbo pump and achievement of a 10^{-8} mbar range pressure within about 12 h. The sample holder on the chamber allowed subsequent treatment of four different Au chips. One of these was used to test and optimize the electron-gun performance and the other three positions for the measurements. Irradiation of a specified area on the chips was achieved by means of Mo masks. They were positioned closely (∼0.2 mm) in front of each Au chip, being electrically isolated from it. The masks had an opening (4 mm diam.) at the centre which enclosed the array of the 8×8 pattern. The electron doses ware determined by integrating the current at the sample passing thought the opening in the mask using an ampèremeter (Keithley 6485), a PC-card (NI 6014) and a self written Lab View software. The electron gun

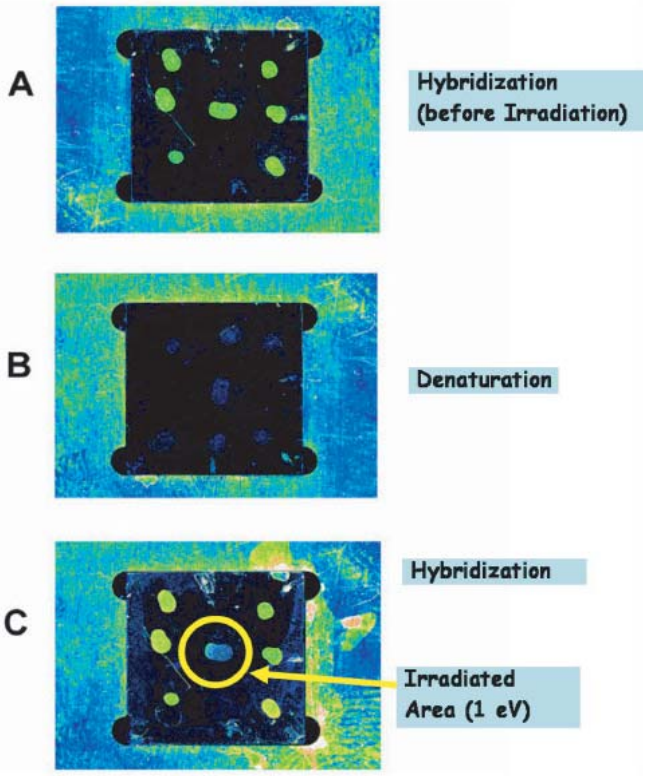

Fig. 2. False colour fluorescence images of oligonucleotide islands (bright spots) on a gold surface (dark area). DNA was deposited by a pipette. (A) hybridized oligonucleotides before electron bombardment, (B) the same sample after denaturation; (C) rehybridization after electron bombardment (only the indicated area was exposed to 1 eV electrons, see text).

was equipped with a BaO emitter (Heatwave Labs, USA) and had electron energy resolution of ∼0.25 eV (as determined from i-E derivative at the current onset). The gun was placed about 10 cm from the Au chips for uniform exposure and operated at 600 to 850 nA at the sample. Negative tests were performed (by deflecting the electron beam from the target) to verify that there was no diverse effects of light or heat from the filament.

Following the electron irradiation the Au chips were again hybridized with the complementary strand, washed in 3 × SSC and ultra pure water, dried with nitrogen and scanned again with the Affimetrix scanner. The obtained spot intensities (I_i) were then normalized using corresponding intensities before the electron bombardment (I_i°) and a parameter called relative Genetic Information Content (GIC) was obtained as:

$$\mathrm{GIC} = \langle \Sigma_{8\times8}(I_i/I_i^\circ)\rangle / \langle \Sigma_{5\times5,4\times4}(I_i/I_i^\circ)\rangle$$

where the sum $\Sigma_{8\times8}$ assigns the spots in the center of the gold chips (electron irradiated) and the sum $\Sigma_{5\times5,4\times4}$ all the spots at corners of the chip (non irradiated).

3 Results and discussion

In this part of the work we first discuss the reasons for using these particular oligonucleotides. We then present the typical macroarray images and the set of data concerning the changes observed in the images upon variation of electron doses. We then compare the fluorescence results with the relevant gas-phase data concerning negative ion mass spectroscopy. Finally, we discuss the implications of the results with respect to the possible damage to the human genome.

Oligonucleotides composed solely from thymine (T) were chosen for this study for a number of reasons. First of all, its immobilization at a gold surface has been investigated with a number of techniques such as X-ray photoelectron spectroscopy (XPS) [13], infrared spectroscopy [14], neutron diffraction [15] and electrochemistry [16]. There is a reasonable agreement between these studies concerning the saturation surface coverage (about 10^{13} molecules/cm^2 [17]). Furthermore, the length of oligonucleotides used in this study (dT-25 mer) has been reported to minimize the interaction with the surface [18] and, on the other side, to provide a sufficiently stable duplex complex resulting in strong fluorescent signals. Finally, a number of important information concerning interaction of gas phase thymine [6,7] and thymidine [19], as well as sugar [10], with low energy electrons exist in the literature.

In Figure 3 typical fluorescence images before and after electron irradiation of a gold chip containing different T_{25} sub microarrays are shown. The spots were deposited precisely with a uniform spot-to-spot spacing of 250 μm. Only the 8 × 8 microarray in the centre of the chip was exposed to electrons. The 5 × 5 sub array (also used to determine the orientation of the chip) and the three 4 × 4 DNA microarrays at the corners were not exposed to electrons (being protected by a mask).

Otherwise they were subjected to the same wet-chemistry/vacuum treatment as the 8 × 8 microarray at the centre. This means that the statistical analysis of the data compares the reactivity of the 64 DNA probes towards electrons with 73 control DNA probes. The images in Figure 3 clearly demonstrate the decomposition of the immobilized oligonucleotides by 1 eV electrons. At this point we recollect the recent gas-phase results concerning thymidine [19] which show that the DEA fragmentation reactions take place in the energy range 0–3 eV. We note here briefly that the degradation of alkanethiol molecules adsorbed on gold, such as HS-$(CH_2)_5CH_3$, has a threshold at 5–7 eV [20] and that a decomposition of the thiol link of the DNA probes can be excluded.

In Figure 4 we show the dependence of the fluorescence intensity on the electron doses at electron energy of 1 eV. It is immediately clear that the fluorescence microarray technology applied in this study is very sensitive in detecting the low energy electron damage to DNA. However, a number of important questions have still to be answered. Initially the damage to oligonucletides increases very rapidly (strong initial decrease in the fluorescent intensity as represented by GIC values), followed by a

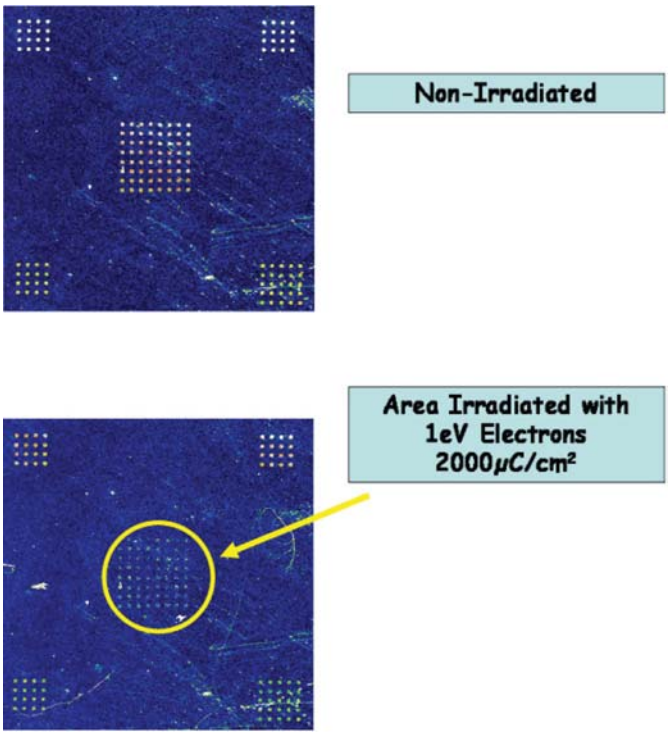

Fig. 3. False colour fluorescence images of the non-irradiated (above) and irradiated (below) Au chip containing different dT_{25} sub microarrays (bright spots). For the chip below only the area within the indicated circle was irradiated with 1 eV electrons.

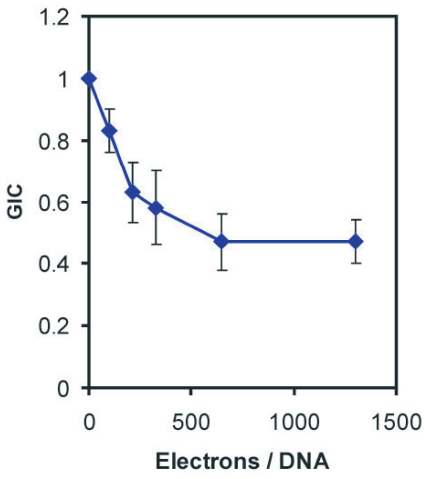

Fig. 4. Electron doses dependence of the damage to dT_{25} oligonucleotides (1-GIC values) by electrons of 1 eV energy (see text for details). Surface concentration of 10^{13} oligonucleotide/cm^2 is assumed [17].

saturation at electron doses above about 500 electrons per immobilized oligonucleotide. The origin of this saturation behaviour is presently unclear, but it might concern self shielding of oligonucleotides, that is a limiting penetration depth of electrons into the adsorbed oligonucleotide layer. It is important to note that so far the mechanism how low energy electrons damage DNA is not known. Possible scenarios are electron capture by the nucleotide bases and transfer of the excess charge to the backbone [21] or electron attachment directly to the backbone [10,19].

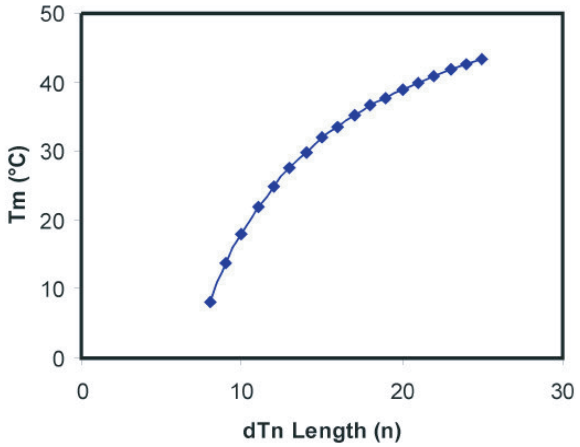

Fig. 5. Primer annealing temperature (the temperature up to which the double strand $(T\text{-}A)_n$ are stable) as a function of dT_n oligonucleotide length [22]. The annealing temperature correlates directly with the fluorescent signal in this study.

We also point that the fluorescence signal correlates with the length of the oligonucleotides. This correlation is non-linear since the hybridization efficiency and the stability of the DNA duplex increases non-linearly with length (Fig. 5). In other words, a strand break induced at the middle of an oligonucleotide will result in a stronger decrease of the fluorescence signal than a break at the exposed end. It therefore appears that the efficiency to induce a break and significantly reduce the length of the oligonucleotides is much higher at the beginning of the exposure to electrons. At that time longer oligonucleotides are present on the surface compared to later stages where fragmentation reactions already have taken place. A more precise description and quantification of the damage to the oligonucleotides requires further fluorescence studies with model oligonucleotides of different lengths and with intentionally synthesized internal mismatches at specific positions. Such studies are in progress.

It is worth to compare the fluorescence data of this study with the work concerning the formation of single stranded breaks by low energy electrons in plasmid DNA as obtained from the electrophoresis data [1]. There it was found that the formation of a single stranded break requires on average a few hundred electrons, in agreement with the data in Figure 4. Furthermore, in the initial, linear low electron doses part of the curve in the Figure 4, a linear fit would be proportional to a total cross-section of degradation (i.e. loss of GIC/incident electron), if we assume the loss of GIC is due to the total oligonucleotide damage from all possible reaction channels (sugar-phosphate backbone fragmentation, base abstraction, reaction of radical sites created on oligonucleotides, etc.). From it we estimate (assuming 10^{13} oligonucleotides/cm^2 [17]) a value for the total cross-section of about 1.5×10^{-16} cm^2. This is in a reasonable

good agreement with the value obtained in previous measurements [23] of neutral fragment desorption from single stranded 12-mer oligonucleotides chemisorbed on Au via a thiol modification of the phosphate groups of oligonucleotides (5×10^{-17} cm^2 at electron energy of 12 eV).

In conclusion, the fluorescence method is able to detect very sensitively low energy electron damage to single stranded DNA. The DNA damage and the concomitant loss of genetic information (loss of the ability to bind a complementary strand) occur at surprisingly low electron doses. A rather significant loss of the genetic information occurs upon interaction of only a few hundred electrons with a single stranded DNA containing 25 nucleotides. Therefore, single stranded DNA and RNA are much more endangered during replication, transcription or even translation stages than the current radiation damage models envisage. Finally we mention that, in the light of role of proteins in protecting DNA against radiation [24], this type of studies should be extended to DNA-protein complexes.

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