DNA damage by soft X-ray exposure at oxygen K-edge

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Abstract. In order to obtain detailed insights into the physicochemical mechanism of DNA damage induction in terms of photoabsorption modes, we have prepared thin DNA films of closed circular plasmid (pUC18) on a cover slip without any additives. Using this film, we have performed preliminary experiments by exposing to soft X-rays with energies around oxygen K-shell ionization threshold. The DNA damage yields of strand breaks and base lesions or AP sites were quantified by biochemical treatments. We confirmed that the DNA film can work as a specimen irradiation. The DNA damage yields induced by $\pi^*$ excitation of a K-shell electron of oxygen atoms in DNA were significantly larger than those for oxygen K-ionization.

1. Introduction

The radiobiological effects, such as mutation induction, are thought to arise from formation of the DNA damage, e.g. nucleobase lesions, single- or double-strand breaks, or abasic sites (AP sites). In order to obtain more detailed insights into the physicochemical mechanism of the damage induction in terms of photoabsorption-modes, we have tried site selective absorption in DNA using high resolution soft X-rays [1]. Fujii et al. exposed soft X-rays to a thin DNA film in a high vacuum chamber [2]. They suggested that the yields of DNA damage strongly depend on the ionization of nitrogen or oxygen atoms in DNA, particularly much higher yields of base lesions above oxygen K-edge. Their DNA films, however, contained buffer solutes with a few ten % (w/w) to stabilize DNA even in a vacuum. Thus the damage induction by irradiation potentially included some effects from the solutes. The fact makes it difficult to examine specific effects of the resonant photoabsorption-modes, excitation of the $1s$ electron to an antibonding $\pi^*$ or $\sigma^*$ orbital, because of their overlapping energies with those of the buffer solutes. In order to eliminate the effect from the additives, it is crucial to prepare a thin film that is solely composed of DNA even though the DNA might be destabilized to some extent.

In this study, we have tried to prepare thin DNA films of closed circular plasmid without any additives. We tested the stability of the DNA in the film, and also performed preliminary soft X-ray irradiation experiments. The yield of DNA damage produced by soft X-ray irradiation around oxygen K-shell ionization threshold were determined, and compared with our previous data for DNA films including buffer solutes as additives.

2. Materials and Methods

2.1. Preparation of Plasmid DNA films

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Plasmid DNA (pUC18, 2686 bp) was purified from *E. coli* strain HB101 using a plasmid purification kit (QIAfilter plasmid Mega kit, QIAGEN, Japan). The plasmid, which is over 95% in the intact closed circular form, was subsequently stored at -20 °C in TE buffer (10 mmol dm⁻³ Tris, 1 mmol dm⁻³ EDTA, pH 8.0) at a concentration of about 1 mg/ml. Then we removed the solutes from the plasmid DNA solution using a filter kit for PCR purification (QIAquick PCR Purification Kit, QIAGEN), and the plasmid DNA was diluted with sterilized water to give a final DNA concentration of 0.1 µg/µl. Five µl aliquots of this plasmid solution were spotted onto various substrates and dried for 30 min in air. We tested a cover slip, silicon wafer evaporated with or without gold, copper plate and graphite plate as substrates for the sample spotting.

### 2.2. Irradiation of soft X-ray beam to the plasmid DNA films

Irradiation was carried at the soft X-ray beamline (BL23SU) in SPring-8 (Hyogo, Japan). Monochromatic soft X-rays were obtained using a varied line spacing grating. The irradiation of the sample films was carried out at the selected energies at an oxygen K-shell (1s) π* resonance peak (531.6 eV). We also used soft X-rays of 560 eV that is completely larger than the oxygen 1s binding energy (543.1 eV)[3]. The plasmid DNA film on a substrate was placed in a pre-chamber (~10⁻⁵ Pa) for 1 h, and was then transferred to a high vacuum irradiation chamber (~10⁻⁷ Pa). To obtain uniform irradiation, the sample holder was moved up and down, and also right to left in the vacuum chamber using motor-drive manipulators as described in our previous report [2]. The photon flux of the beam measured using a photodiode (AXUV-100, International Radiation Detectors Inc., USA) at the sample position was almost constant in the order of 10¹⁷ photons per sample. The absorbed doses are estimated based on the photo-absorption cross sections and the atomic composition of the sample. Two independent irradiation experiments were performed.

### 2.3. Treatment of the irradiated DNA samples and gel electrophoresis

After soft X-ray irradiation, the plasmid DNA film was recovered with 15 µl of 40 mM HEPES buffer (pH 7.6) for each sample, and kept at -20 °C until the biochemical treatment to quantify DNA damage. Sixty µl aliquots of the irradiated samples were mixed with enzymatic reaction buffer (50 µl of HEPES buffer with 0.5 mM dithiothreitol (DTT, Nacalai Tesque, Japan), and 0.21 mg/mL bovine serum albumin (BSA). These samples were then incubated at 37 °C for 0.5 h in the "absence" or "presence" of enzymes. The former treatment was performed to examine the yield of promptly induced SSB, and latter one to examine the yields of base lesions or AP site. The heat treatment did not cause additional SSBs (data not shown). We used three kinds of enzymes, formamidopyrimidine-DNA glycosylase (Fpg), endonuclease three (Nth), or endonuclease four (Nfo) to transform damage to an additional SSB by their glycosylase activities. Nth protein excises mainly ring-saturated pyrimidines (e.g., 5, 6-dihydrothymine (DHT)), thymine glycol, and AP sites. Fpg protein excises mainly 2,6-diamino-4-hydroxy-5-N-methyl formamidopyrimidine, 7,8-dihydro-8-oxo-2'-deoxyguanine (8-oxoGua), and AP sites. Nfo protein excises various AP sites. After the excision of the damage, a SSB is formed at the damage site. Purified enzymes Nth, Fpg, and Nfo were purchased from NEB (New England Biolabs Japan, Japan). The optimal concentrations of the enzymes used for the treatments were determined in our previous study [2].

After incubation with or without enzymes, 2.5 µL of 0.5 M EDTA (pH 8.0) was added to the sample solutions and kept at 5 °C on ice to terminate the enzymatic reactions. For gel electrophoresis, 2.5 µL of the loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, and 30% glycerol) was added to the samples, which were placed into the wells of a 1% agarose (Type 1-A, Sigma-Aldrich Japan, Tokyo, Japan) gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) at pH 7.1 and electrophoresed at 1.4 V/cm for 16.7 h at 5.6 °C.

### 2.4. Quantification of yields of SSB and DSB in irradiated plasmid DNA

After electrophoresis, the gel was stained with 30 µL of ethidium bromide (Bio-Rad Japan, Tokyo, Japan) (10 mg/mL) in 600 µL of TBE buffer for 1 h. The positions of closed circular, open circular, and linear forms of the plasmid DNA were separately visualized with a charge-coupled device (CCD,
Ettan DIGE, GE Healthcare UK Ltd., England) and analyzed with ImageQuant TL software (GE Healthcare UK Ltd., England). The dose response was determined from the loss of intact closed-circular DNA resulting from the strand breaks promptly induced by irradiation, or enzymatically induced strand breaks at base lesions or AP sites. The \( D_{37} \)-values, a dose giving 37\% of the remaining DNA in the closed circular form, was obtained from the dose responses. These values, indicators for induction of any kinds of SSBs, reveal the radiation dose required to give an average of one SSB per plasmid DNA. According to the \( D_{37} \)-value, a yield of SSB \( \frac{n(\text{SSB})}{\text{Gy/Da}} \) was calculated from equation (1) assuming that the average mass of a base pair is 650 Da and that pUC18 DNA contains 2686 base pairs.

\[
n(\text{SSB}) = \frac{1}{2686 \times 650 \times D_{37}} \tag{1}
\]

The yields of lesions revealed by the enzymatic treatments, described as number of enzyme sensitive sites, \( n(\text{ESS}) \), observed as additional SSBs were obtained with Equation 2.

\[
n(\text{ESS})_{\text{Nth (or Fpg, Nfo)}} = n(\text{SSB})_{\text{Nth (or Fpg, Nfo)}} - n(\text{SSB})_{\text{prompt}} \tag{2}
\]

3. Results and Discussion

Most of the materials tested for the substrate of the sample gave a pronounced degradation effect to plasmid DNA. Over 50\% of the sample plasmid DNA molecules were suffered strand breaks by spotted on the surfaces, or kept in a vacuum for several hours. Only the cover slip gave us sustainable results because over 60\% of the plasmid kept their intact closed circular form in a control condition without exposure as shown in Figure 2. Thus hereafter we adopted the DNA films on the cover slip as a specimen irradiation. A ring-shape film of DNA of 4.0 mm diameter was formed on the cover slip. X-ray absorption near edge structure (XANES) spectrum determined by transmission of soft X-rays in a calf thymus DNA film [4] is shown in Figure 1 with the energies chosen for exposure in this study. The energies of the oxygen \( \pi^* \) excitation peak (531.6 eV) and above ionization threshold (560 eV) are give the sample DNA a similar photoabsorption cross section. Using an atomic force microscope the thickness of the sample film was determined to be about 200 nm. We also ensured absence of charge up of the sample because the XANES spectrum obtained as a total electron current in the sample film did not get distorted by the soft X-ray exposure.

A typical example of the dependence of the amount of closed circular DNA on absorbed dose is shown in Figure 2 for the samples exposed to soft X-rays at the energy of the \( \pi^* \) excitation. The fractions of the intact plasmid DNA exponentially decreased with increasing dose. These data ensure that soft X-rays considerably penetrated in the sample film and the most of the plasmid molecules

![Figure 1. Photoabsorption spectrum of calf thymus DNA film. The arrows show the energy chosen for this study.](image1)

![Figure 2. Dependence of the loss of intact (closed circular) plasmid DNA on soft X-ray (531.6 eV) dose after exposure. The straight lines are drawn by the least square method.](image2)
were exposed to the soft X-rays uniformly. If the thickness of the sample film much higher than penetration depth of the soft X-rays and the molecules located in the back of the film are not adequately irradiated, resulting that the dose response does show a saturation in the higher dose region. The post-irradiation incubation with the enzymes results in greater loss of closed-circular DNA than seen in the absence of an enzyme treatment for a given dose.

From the obtained dose-dependences, the yields of prompt SSB and base lesions visualized by the biochemical treatments were calculated from the $D_{37}$-values (see Equation 1 and 2). These yields are tabulated in Table 1 with our previous data [2]. The yields of SSB and Nth sensitive site are larger than those reported by Fujii et al. [2], indicating that the buffer solutes may act to protect DNA chemically active species to produce these DNA lesions. All the yields of DNA damage examined for 531.6 eV X-ray irradiation are significantly larger than those for 560 eV. This indicates that the $\pi^*$ excitations of an oxygen 1s electron to an antibonding orbital may effectively cause chemical bond breakages resulting induction of certain DNA damage. Impact of ejected photo- or Auger electrons to surrounding DNA is also thought to contribute the damage induction. It is particularly worth noting that the $\pi^*$ excitation does emit less electrons than oxygen K-ionization, only an Auger electron to be ejected, hence the contribution of the $\pi^*$ excitation is more apparent in the damage induction than the secondary effect of electron impact. In future the effect of $\sigma^*$ excitation of oxygen atoms in DNA will be further studied using the experimental procedure established in the present study.

Table 1. Yields of SSB and ESS induced in pUC18 plasmid DNA by soft X-ray irradiation.

| DNA Damage               | Yield ($\times10^{-11}$ SSB or ESS/Gy/Da) |
|-------------------------|-----------------------------------------|
|                         | 531.6 eV (O-K $\pi^*$ resonance)       | 560 eV (O-K ionization) |
|                         | 560 eV (O-K ionization)$^a$             |
| n(SSB) Single strand breaks | 11.6                                    | 7.3                   | 2.8 |
| n(ESS$_{Nth}$) Pyrimidine lesions and AP sites | 12.4                                    | 7.4                   | 5.8 |
| n(ESS$_{Fpg}$) Purine lesions and AP sites | 6.3                                     | 4.0                   | 4.5 |
| n(ESS$_{Nfo}$) AP sites | 4.5                                     | 3.4                   | -   |

$^a$ Fujii et al. 2009.

Reference
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