DNA DAMAGE INDUCED BY SECONDARY ELECTRONS AND X-RAYS

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To Xifeng, Augusta, and Angela Li...
# TABLE OF CONTENTS

LIST OF FIGURES AND TABLES.................................................................iv

LIST OF ABBREVIATIONS AND SYMBOLS.................................................ix

ABSTRACT.......................................................................................xii

I. INTRODUCTION..............................................................................1

I.1. Radiation and Health.................................................................1

   I.1.1. Radiation protection............................................................1

   I.1.2. Applications of radiation in medicine.......................................2

   I.1.3. Radiobiology, dose fractionation and treatment planning............4

I.2. Effects of radiation on DNA.......................................................5

   I.2.1. Types of DNA damage.........................................................5

   I.2.2. Direct vs indirect effects.......................................................5

   I.2.3. DNA damage induced by low energy electrons (LEE)...............8

      I.2.3.1 Interaction of LEE with condensed matter.......................9

      I.2.3.2 Interaction of LEE with plasmid DNA.............................10

      I.2.3.3 Interaction of LEE with oligonucleotides.......................12

I.3. X-ray induced electron emission from metals.............................15

I.4. Research Project.....................................................................17
II. RESULTS .................................................................................................................. 19

II.1. Article No. 1: Dosimetry of ultrasoft X-rays (1.5 keV Al_{k\alpha}): using radiochromatic films and color scanners................................................................. 19

II.2. Article No. 2: DNA inter-duplex crosslinks induced by Al_{k\alpha} x-rays under vacuum.................................................. 51

II.3. Article No. 3: Comparison between X-ray photon and secondary electron damage to DNA in vacuum............................................................. 76

II.4. Article No. 4: Enhanced DNA damage by secondary electron emission from a tantalum surface exposed to clinical X-rays........................................... 96

II.5. Article No. 5: Induction of strand breaks by low energy electrons (8-68 eV) in a self assembled monolayer of oligonucleotides: effective cross sections and attenuation lengths......................................................... 123

III. DISCUSSION ........................................................................................................ 142

III.1. Is it possible to convert a quantum yield obtained by LEE beam experiments to a G value? ................................................................. 142

III.2. Why are low energy secondary electrons more efficient than X-ray photons for induction of DNA SSB and DSB? ................................................................. 142

III. 3. Are G values for the induction of DNA SSB and DSB for SE emission from Ta exposed to Al_{k\alpha} X-rays applicable to SE induced by X rays in a general biological environment? ......................................................... 144
III.4. Does ionizing radiation cause DNA interduplex CL in cells? .......................144

III.5. Back-scattered SE from metal surfaces exposed to clinical X-rays enhance DNA SSB, DSB, and CL. What is the potential impact of this enhancement on the clinical practices of X-ray diagnosis and radiotherapy? ........................................147

III.6. The application of effective cross sections per nucleotide for DNA SB by LEE to radiation protection and radiotherapy ..................................................148

IV. CONCLUSION/PERSPECTIVE ..................................................................149

V. ACKNOWLEDGEMENTS ..........................................................................152

VI. REFERENCES ..........................................................................................153

VII. APPENDIX ..............................................................................................161

VII.1. γ-radiolysis of aqueous solutions of DNA as a tool to test the purity of plasmid DNA ........................................................................................................161

VII.2. The stability of plasmid DNA in UHV ......................................................165

VII.3. The stability of plasmid DNA in air ........................................................166

VII.4. Why is it better to label oligonucleotides with $^{32}$P before exposure to an electron beam? ........................................................................................................167

VII.4.1. The effect of oligonucleotide lengths on their recovery from a Sephadex G50 column ........................................................................................................168

VII.4.2. The effects of the length of oligonucleotides on the efficiency of 5’-end $^{32}$P labeling using T4 polynucleotide kinase ..................................................169
# LIST OF FIGURES AND TABLES

1. Table I.1  Some sources and amounts of ionizing radiation exposure ..............4

2. Table II.1.1  Density and composition of HD-810 films.........................44

3. Table II.1.2  The effect of colour adjustment and channel choice on the responses of HD-810 films to the absorbed doses of $\gamma$-rays to water ................................................................. 45

4. Table II.1.3  Mass energy absorption coefficients of water, three layers of HD-810 films and attenuation of photon intensity by each component at three levels of photon energy ...............46

5. Figure II.1.1  Plot of ABS of HD-810 films exposed to $\gamma$-rays and soft x-rays (effective energy of 14.8 keV) versus the absorbed doses in water ................................................................. 47

6. Figure II.1.2  Plot of ABS of HD-810 films exposed to $\gamma$-rays versus the absorbed doses in water ................................................................. 48

7. Figure II.1.3  Plot of ABS of HD-810 films exposed to ultrasoft x-rays (photon energy of 1.5 keV) versus the irradiation time as a function of emission currents ........................................ 49

8. Figure II.1.4  Plot of the mean absorbed dose to the active layer of HD-810 films exposed to Al$_{K\alpha}$ ultrasoft X-rays versus the irradiation time as a function of the emission currents ........... 50

9. Scheme  Paths for the formation of circular (C), linear (L) and II.2.1 crosslinked (S-S, S-C, C-C) DNA ........................................... 58

10. Figure II.2.1  Neutral agarose gel electrophoresis of pGEM®-3Zf(-)
plasmid DNA: effects of X irradiation and digestion with
EcoR1 and PvuI restriction enzymes

Figure II.2.2 Alkaline agarose gel. Lanes from the left: 1, lambda
DNA-HindIII digest; 2, 1-kb DNA ladder; 3,
onirradiated pGEM®-3Zf(-) plasmid DNA-EcoR1 digest;
4, X-irradiated 3Zf(-) plasmid DNA-EcoR1 digest

Figure II.2.3 The dependence of the formation of circular (C), crosslink
1 (C-C), crosslink 2 (S-C) and linear (L) DNA as well as
the loss of supercoiled (S) and concatameric (CM) DNA
on mean absorbed dose in DNA

Figure II.2.4 The effect of mean absorbed dose rate on the formation of
circular, crosslink 1, crosslink 2 and linear DNA and
the loss of supercoiled and concatameric DNA

Figure II.3.1 Energy spectra of Al Kα X-ray induced secondary
electron emission from tantalum

Figure II.3.2 Agarose gel electrophoresis of DNA following irradiation
by Al Kα X-rays as a monolayer on tantalum foil

Figure II.3.3 X-ray exposure curves for the loss of supercoiled (a) and
concatemeric (b) DNA as well as the formation of circular
(c), linear (d), cross-link form 1 (e), and cross-link form 2
(f) DNA when DNA was irradiated in a thick film and a
monolayer

Table II.3.1 G values for formation and loss of different forms of
DNA induced by X-ray photons and secondary electrons ........................................... 88

18 Figure II.3.4 LEE enhancement factor (LEE EF) as a function of photon energy for SSB and DSB in monolayer DNA deposited on tantalum ......................................................................................................................... 91

19 Table II.4.1 G values for the formation of different forms of DNA induced by X-ray photons and secondary electrons at two DNA hydration levels ......................................................................................................... 108

20 Figure II.4.1 A photo of the home-made box used for X-ray irradiation ........... 120

21 Figure II.4.2 Dependence of the net optical density of radiochromatic films on the mean X-ray absorbed dose in the active layer: the effect of X-ray induced electron emission from tantalum ......................................................................................................................... 121

22 Figure II.4.3 X-ray exposure curves for the formation of circular (a), linear (b), crosslink 1 (c) and 2 (d) DNA as well as the loss of supercoiled (e) and concatemeric (f) DNA in thick and thin films of DNA in air ........................................................................................................... 122

23 Table II.5.1 Attenuation length and effective cross section for SB in SAM of oligonucleotides chemisorbed on gold as a function of electron energy ........................................................................................................... 136

24 Figure II.5.1 Representation of a self-assembled monolayer (SAM) of a thiolated oligonucleotide chemisorbed on a gold substrate and its exposure to an electron beam ........................................................................ 137

25 Figure II.5.2 Dependence of total yield of LEE-induced 5’-
oligonucleotide fragments on the amount of 58 eV electrons incident at a constant current density of $1.2 \times 10^{12}$ electrons cm$^{-2}$ s$^{-1}$ ........................................ 138

26 Figure II.5.3 Dependence of yield of LEE-induced 5'-oligonucleotide fragments on their length for electron energies of 8, 28 and 68 eV. The inset shows the dependence of the attenuation length on LEE energy ......................... 139

27 Figure II.5.4 Denaturing polyacrylamide gel of oligonucleotides...... 140

28 Figure II.5.5 Energy dependence of the effective cross section for the induction of strand breaks by LEE in self-assembled monolayer of 3'-thiolated oligonucleotides ..................... 141

29 Figure III.1 Levels of Chromatin Packing ........................................ 146

30 Figure VII.1 The dependence of the formation of circular DNA (SSB) on the $\gamma$-absorbed dose in aerated aqueous solution containing 80 ng $\mu$L$^{-1}$ plasmid DNA and 10 mM phosphate buffer, pH 7.8 ............................. 162

31 Figure VII.2 The dependence of the formation of circular DNA (SSB) on the concentration of NaHCO$_3$ for the $\gamma$-radiolysis of aerated aqueous solution containing 17 ng $\mu$L$^{-1}$ plasmid DNA, 10 mM phosphate buffer (pH 7.8) and NaHCO$_3$. The absorbed dose is 0.9 Gy. The inset shows the dependence of the induction of SSB on the absorbed dose in aerated aqueous solution containing 28 ng $\mu$L$^{-1}$ plasmid
DNA, 0.6 M NaHCO₃ and 10 mM phosphate buffer, pH 7.8.................................................................  .......163

32 Figure III.3 The effects of the thickness of DNA films, tantalum surfaces and storage time of DNA in UHV on the stability of plasmid DNA.........................................................  .......165

33 Figure III.4 The effects of DNA freezing methods, DNA concentrations and storage time on the stability of plasmid DNA in air, at 4°C and relative humidity of 45%..............  .......166

34 Figure III.5 Effects of the length and total concentration of oligonucleotides on their recovery ratio from a Sephadex G50 column...............................................................  .......169

35 Figure III.6 Effects of the length of oligonucleotides on the relative efficiency of 5’-end ³²P labeling using T4 polynucleotide kinase.................................................................  .......170
LIST OF ABBREVIATIONS AND SYMBOLS

ABS  the net optical density
AL   attenuation length
BL   the active layer
C    relaxed circular plasmid DNA
CAT scans (CT)computerized tomography
C-C  covalently crosslinked DNA consisting of two circular plasmids
CLs (CL) interduplex crosslinks; crosslinks
CM   a concatamer of two supercoiled plasmids
CM-SSB a concatamer of one supercoiled and one relaxed circular plasmid
CM-2SSB a concatamer of two relaxed circular plasmids
DD   dipolar dissociation
DEA  dissociative electron attachment
DSB  double strand breaks
dd H₂O distilled and de-ionized water
E    energy
e_{aq}  hydrated electrons
ESD  electron stimulated desorption
EF   enhancement factor
F1   fragment 1, 1350 bps, resulting from Pvu I digestion of pGEM®-
     3Zf(-) plasmid
F2   fragment 2, 1847 bps, resulting from Pvu I digestion of pGEM®-
     3Zf(-) plasmid
F1-F1  covalently crosslinked DNA consisting of two F1
F1-F2  covalently crosslinked DNA consisting of one F1 and one F2
F2-F2  covalently crosslinked DNA consisting of two F2
G     G values
HD-810 films  GAFCHROMIC® HD-810 radiochromatic dosimetry films
HVL   half-value layer
IAEA  International Atomic Energy Agency
ICRP  the International Commission on Radiological Protection
IMFP  inelastic mean free paths
J     incident electron current density
L     linear DNA
LEE (LEEs)  low energy electrons
LEEEF  LEE enhancement factor
LNT   a linear non-threshold model
MDSB  Multiple double strand breaks
MW    molecular weight
NCRP  the U.S. National Council of Radiation Protection and Measurements
‘OH   hydroxy radicals
OligoS thiolated oligonucleotide
5’-OligoS-F  5’-oligonucleotide fragments
PET   positron emission tomography
S     supercoiled plasmid DNA; average pixels
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$s$</td>
<td>average pixels</td>
</tr>
<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
</tr>
<tr>
<td>SB (SBs)</td>
<td>strand breaks</td>
</tr>
<tr>
<td>S-C</td>
<td>covalently crosslinked DNA consisting of one supercoiled and one circular plasmid</td>
</tr>
<tr>
<td>SD</td>
<td>the standard deviation</td>
</tr>
<tr>
<td>SE (SEs)</td>
<td>secondary electrons</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
</tr>
<tr>
<td>S-S</td>
<td>covalently crosslinked DNA consisting of two supercoiled plasmids</td>
</tr>
<tr>
<td>SSB</td>
<td>single strand breaks</td>
</tr>
<tr>
<td>SSD</td>
<td>source surface distance</td>
</tr>
<tr>
<td>THORP</td>
<td>a titanium hollow-screw osseointegrating reconstruction plate</td>
</tr>
<tr>
<td>UHV</td>
<td>ultra high vacuum</td>
</tr>
<tr>
<td>USX</td>
<td>ultrasoft X-rays</td>
</tr>
<tr>
<td>XPS</td>
<td>analytical X-ray photoelectron spectrometer</td>
</tr>
<tr>
<td>W</td>
<td>work function</td>
</tr>
<tr>
<td>$\phi_0$</td>
<td>the incident fluence rate of photons</td>
</tr>
<tr>
<td>$\Gamma$</td>
<td>the number of water molecules per nucleotide</td>
</tr>
<tr>
<td>$\eta$</td>
<td>the quantum yield of secondary electrons</td>
</tr>
<tr>
<td>$\mu/\rho$</td>
<td>mass attenuation coefficient</td>
</tr>
<tr>
<td>$\mu_{en}/\rho$</td>
<td>mass energy absorption coefficient</td>
</tr>
<tr>
<td>$\sigma_{eff}$</td>
<td>effective cross section</td>
</tr>
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Dommages induits par les électrons secondaires et les rayons X

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Les rayons X sont couramment utilisés pour le diagnostic médical et la radiothérapie. En ce qui concerne leur effet léthal, c’est principalement l’ADN cellulaire qui est ciblé. L’interaction des rayons X avec l’ADN produit des photoélectrons, des électrons Auger et Compton, ainsi qu’une quantité importante d’électrons secondaires (SE), qui ont pour la plupart une énergie inférieure à 70 eV. Le but de cette thèse est de comparer les dommages à l’ADN induits par les SE et les rayons X.

Une méthode dosimétrique a été développée afin de mesurer la dose absorbée de rayons X Al$_{\alpha}$ (1.5 keV) dans une chamé à ultra-vide (UHV) en utilisant des films de dosimétrie radiochromatique et un scanner couleur. Cela a permis de définir la valeur de la dose absorbée dans des échantillons biologiques secs irradiés sous UHV, permettant ainsi une comparaison directe du dommage à l’ADN induit par les rayons X Al$_{\alpha}$ et les électrons secondaires.

L’induction de ponts covalents (dimères) interbrins dans l’ADN (CL) par les rayons X de clinique (14.8 keV) dans l’air a été détectée par digestion avec des enzymes de restriction, suivi par une d’électrophorèse alcaline. La valeur G pour la formation des CL dans l’ADN sous UHV par les rayons X Al$_{\alpha}$ est de 16 ± 4 nmol J$^{-1}$, soit 2.8 fois plus que les cassures double brins (DSB). La valeur G(CL) dans l’air pour les rayons X doux (14.8 keV) est 3 ± 1 (à $\Gamma \approx$6) et 3.2 ± 0.4 ($\Gamma \approx$21) nmol J$^{-1}$, soit 61 et 89% des valeurs pour les DSB, respectivement. Ces résultats suggèrent que les CL sont un type important de dommage à l’ADN pour la radiobiologie, possiblement aussi important que les DSB.
Une nouvelle méthode d’étude des dommages induits par les SE, comparativement à ceux produits par les rayons X Al, dans des conditions expérimentales identiques, a été développée. Cette méthode utilise des films minces ou épais d’ADN, déposés sur du tantale. Les rayons X induisent l’émission de SE à partir du tantale, et fournissent donc la source de SE sous UHV. La limite inférieure des valeurs G pour les SSB et DSB induits par les SE a été évaluée à : $86 \pm 2$ et $8 \pm 2$ nmol J$^{-1}$, respectivement. Ces valeurs sont 1.5 et 1.6 fois plus élevées que celles obtenues pour des photons de 1.5 keV.

L’augmentation des dommages dans l’ADN sous conditions atmosphériques par les SE induits par les rayons X cliniques (14.8 keV) à partir du tantale ont été étudiées. Le facteur d’augmentation pour les SSB, DSB et les CL induits par les SE comparativement aux rayons X a été calculé à : $5.0 \pm 0.8$, $3.6 \pm 1.0$ et $9 \pm 4$ à $\Gamma \approx 6$ et $8 \pm 1$, $9 \pm 2$ et $5.0 \pm 1.4$ à $\Gamma \approx 21$, respectivement. Cette étude fournit une base moléculaire à la compréhension de l’effet accru causé par l’interface biologique durant l’examen diagnostique et la radiothérapie utilisant des rayons X.

Une monocouche autoassemblée (SAM) d’un oligonucléotide marqué en 5’ au $^{32}$P et thiolé en 3’, chimisorbé sur l’or, a été bombardée par des électrons de basse énergie (LEE) de 8 à 68 eV. Les courts fragments d’oligonucléotides marqués en 5’ produits par les cassures simple brin causées par les LEE ont été séparés en électrophorèse sur gel dénaturant d’acrylamide et quantifiés par balayage laser. Le rendement des oligonucléotides courts (y) diminue exponentiellement avec leur longueur (x), suivant l’équation $y = a \times e^{-bx}$, où a et b sont des constantes, et qui sont reliées à la section efficace moyenne par nucléotide pour les cassures à l’ADN ($\sigma_{\text{eff}}$) et à la distance d’atténuation (AL = 1/b) des LEE, respectivement. AL diminue avec l’énergie des LEE ($2.5 \pm 0.6$nm à 8 eV jusqu’à $0.8 \pm 0.1$nm à 68 eV), tandis que $\sigma_{\text{eff}}$ augmente de $(3 \pm 1) \times 10^{-18}$ à $(5.1 \pm 1.6) \times 10^{-17}$cm$^2$ à l’intérieur de la même gamme d’énergie. La dépendance énergétique de $\sigma_{\text{eff}}$ montre un pic de résonance de $(2.8 \pm 0.9) \times 10^{-17}$ cm$^2$ à 18 eV, superimposé sur une courbe monotone ascendante. Un attachement dissociatif de l’électron sur le squelette sucre-phosphate de l’ADN est proposé pour expliquer ce comportement..
DNA damage induced by secondary electrons and X-rays

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X-rays are widely used in medical diagnosis and radiotherapy. The effects of X-rays on cells are dominated by their actions on the cellular DNA. The interaction of X-rays with DNA produces photoelectrons, Auger electrons, Compton electrons and large quantities of secondary electrons (SE), the majority of which have energies lower than 70 eV. This thesis aims to compare the DNA damage induced by SE and X-rays.

A dosimetry method to measure the absorbed dose of \( Alk\alpha \) X-rays (1.5 keV) in ultrahigh vacuum chamber (UHV) using radiochromatic dosimetry films and a color scanner was developed. This provided the value for the absorbed dose in dry biological samples irradiated under UHV, allowing direct comparison of DNA damage induced by \( Alk\alpha \) X-rays and SE.

The induction of covalent interduplex DNA crosslinks (CL) by X-rays was detected using restriction enzyme digestion and alkaline agarose gel electrophoresis. The G value for CL induction in DNA in UHV by \( Alk\alpha \) X-rays is \( 16 \pm 4 \text{ nmol J}^{-1} \), 2.8 fold that of double strand breaks (DSB). G(CL) in air by soft X-rays (14.8 keV) is \( 3 \pm 1 (\Gamma \approx 6, \Gamma \) is defined as the number of water molecules per nucleotide.) and \( 3.2 \pm 0.4 (\Gamma \approx 21) \text{ nmol J}^{-1} \), 61 and 89 % of those for DSB. These results suggest that CL is an important type of DNA damage, possibly as important as DSB in radiobiology.

A new method of studying SE-induced DNA damage in comparison to \( Alk\alpha \) X-rays under identical experimental conditions was developed, using thin and thick films of
DNA deposited on tantalum. X-ray induced SE emission from the tantalum serves as a source of SE in UHV. The lower limits of $G$ values for DNA SSB and DSB induced by SE were derived to be: $86 \pm 2$ and $8 \pm 2$ nmol J$^{-1}$, respectively. These values are 1.5 and 1.6 times larger than those obtained with 1.5 keV photons.

The enhanced DNA damage by SE emission from tantalum exposed to soft X-rays (14.8 keV) in air was studied as a function of DNA hydration levels. The enhancement factor for SSB, DSB and CL induced by SE vs X-rays were derived to be: $5.0 \pm 0.8$, $3.6 \pm 1.0$ and $9 \pm 4$ at $\Gamma \approx 6$, and $8 \pm 1$, $9 \pm 2$ and $5.0 \pm 1.4$ at $\Gamma \approx 21$, respectively. This study provides a molecular basis for understanding the enhanced interface biological effects during X-ray diagnostic examination and radiotherapy.

A self-assembled monolayer (SAM) of a $5^{'-}{}^{32}$P-labelled $3^{'-}$-thiolated oligonucleotide chemisorbed on gold was bombarded by low energy electrons (LEE) of 8 to 68 eV. Shorter $5^{'-}{}^{32}$P-oligonucleotides produced by LEE-induced single strand breaks were separated with denaturing polyacrylamide gel electrophoresis and quantified by phosphor imaging. The yields of short oligonucleotides ($y$) decrease exponentially with their length ($n$), following the equation $y = a \times e^{bn}$, where $a$ and $b$ are constants, which are related to the average effective cross section per nucleotide for DNA strand break ($\sigma_{\text{eff}}$) and the attenuation length ($\text{AL} = 1/b$) of LEE, respectively. The AL decreases with LEE energies from $2.5 \pm 0.6$ nm at 8 eV to $0.8 \pm 0.1$ nm at 68 eV, whereas $\sigma_{\text{eff}}$ increases from from $(3 \pm 1) \times 10^{-18}$ to $(5.1 \pm 1.6) \times 10^{-17}$ cm$^2$ within the same energy range. The energy dependence of $\sigma_{\text{eff}}$ shows a resonance peak of $(2.8 \pm 0.9) \times 10^{-17}$ cm$^2$ at 18 eV superimposed on a monotonically rising curve. Dissociative electron attachment to the sugar phosphate backbone is proposed to account for this maximum.
I. INTRODUCTION

I.1. Radiation and Health

Radiation is a two-edged sword: its usefulness in medicine is well documented, yet its harmful effects in health are also undisputed.

I.1.1. Radiation protection

Ionizing radiation is a well known cancer risk factor. Most epidemiological evidence such as Japanese atomic bomb survivors and radiobiological studies on DNA damage and repair, chromosome aberrations and cell mutations support a linear non-threshold model (LNT) for the estimation of radiation risk (ICRP 2004). Although LNT is challenged by the superlinear, threshold and hometric hypotheses (Pollycove and Feinendegen, 1999), the International Commission on Radiological Protection (ICRP, 2004), International Atomic Energy Agency (IAEA, 2000), and the U.S. National Council of Radiation Protection and Measurements (NCRP, 2001) all recommend the LNT hypothesis as a defensible basis for radiation protection at low doses and low dose rates.

Radiobiological evidence shows that the induction of DNA double-strand breaks (DSB) and more complex clustered DNA damage is probably the most important mechanism by which ionizing radiation exposure causes radiation carcinogenesis (Goodhead, 1994; Ward, 1995). However, evidence for bystander effects and genomic instability is challenging our thinking about the critical targets for genetic and carcinogenic damage by radiation (Little, 2000).
I.1.2. Applications of radiation in medicine

Radiation is widely used in medical diagnosis and therapy. Radiology, nuclear medicine and radiation oncology form the main medical branches which apply radiation.

Diagnostic radiology mainly makes use of ionizing radiation, nuclear magnetic resonance (e.g. MRI), and ultrasound. X-rays are used for radiography of the chest, bones, joints, teeth and gastrointestinal systems etc; mammography to detect breast cancer in its earliest stages; catheter angiography to examine the heart, blood vessels and blood flow; fluoroscopy, i.e., real-time images that show movement; and finally, computerized tomography (CAT scans), i.e., detailed cross-sectional images of the body (RSNA, 2005). The peak X-ray tube potential for medical diagnosis is usually in the range of 40-140 kV, eg. 40-100 kV for radiography, 20-30 kV for mammography, 40-90 kV for fluoroscopy, 70 kV for oral radiology and 100-140 kV for CAT scans (Novelline, 2004).

Nuclear medicine is concerned with the diagnostic and therapeutic uses of radio isotopes, excluding sealed radionuclide sources for brachytherapy. For example, positron emission tomography (PET) uses neutron deficient radionuclides (e.g. $^{18}\text{F}$, $\tau_{1/2}=109.8$ min; $^{11}\text{C}$, $\tau_{1/2}=20.4$ min) to provide real-time images of biochemical processes. A positron and an electron annihilate, emitting two 511 keV $\gamma$ photons. The $\gamma$ photons are detected by a scintillation camera to produce an image (Townsend 2004). Other examples include imaging the thyroid gland using $^{99m}\text{Tc}$ and $^{123}\text{I}$ (Smith and Oates, 2004) and single photon emission computed tomography (SPECT) using $^{201}\text{Th}$ for the detection of viable myocardium (Bourque et al. 2003).
Radiation oncology (radiotherapy) involves radiation treatment of abnormal tissue
growths, including both malignant and nonmalignant tissues. Radiation sources for
radiotherapy include external beams, eg. X-rays of 30 kV to 25 MV, electron beams of 5-
20 MV, $^{60}$Co and $^{137}$Cs; and internal sealed radionuclides (brachytherapy). More than half
of cancer patients have undergone radiotherapy at some stage during their sickness, either
alone or combined with chemotherapy and/or surgery, either to relieve the pain or to cure
cancer (Wang 2000).

Radiotherapy may be used to treat almost every type of solid tumour, soft tissue
sarcomas, leukemia and lymphoma. The total prescribed dose depends on dose
fractionation protocols, the tumour type and size, the tolerance of the patient, and the
purpose of treatment (eg. palliative or curative). Dose fractionation usually results in a
higher therapeutic ratio by minimizing the damage to healthy tissue but maximizing the
killing of cancer cells. Therapeutic ratio is the corner stone of clinical radiation oncology.
Generally radioresponsive tumours require a lower dose for cure than radio-resistant
tumours. For example, germ cell tumours require a dose of 30-45 Gy in 4 to 5 weeks;
squamous cell carcinoma and adenocarcinoma, 65 to 70 Gy in 7 to 8 weeks; bone and
soft tissue sarcoma, 70 to 75 Gy in 7 to 8 weeks (Wang 2000).

Compared to radiation therapy, the dose for each medical diagnostic examination
is three to four orders of magnitude lower, but is comparable to the annual natural or
 occupational background (Table I.1). Therefore, it is recommended to limit unnecessary
 use of radiation for medical examination and to balance the benefit of the exam vs the
 radiation risk.
Table I.1 Some sources and amounts of ionizing radiation exposure (unless noted, from Mettler and Upton, 1995)

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Effective dose, in mSv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural background (world population)</td>
<td></td>
</tr>
<tr>
<td>Normal background areas</td>
<td></td>
</tr>
<tr>
<td>Cosmic rays</td>
<td>0.38 / year</td>
</tr>
<tr>
<td>Terrestrial rays</td>
<td>0.46 / year</td>
</tr>
<tr>
<td>Radionuclides in tissue</td>
<td>0.25 / year</td>
</tr>
<tr>
<td>Inhaled radionuclides</td>
<td>2.5 / year</td>
</tr>
<tr>
<td>Medical diagnostic (U.S. population)</td>
<td>Per exam</td>
</tr>
<tr>
<td>Skull</td>
<td>0.22</td>
</tr>
<tr>
<td>Cervical spine</td>
<td>0.20</td>
</tr>
<tr>
<td>Chest</td>
<td>0.08</td>
</tr>
<tr>
<td>Cholioangiogram</td>
<td>1.89</td>
</tr>
<tr>
<td>Lumbar Spine</td>
<td>1.27</td>
</tr>
<tr>
<td>Upper gastrointestinal series</td>
<td>2.44</td>
</tr>
<tr>
<td>Abdomen</td>
<td>0.56</td>
</tr>
<tr>
<td>Barium enema</td>
<td>4.06</td>
</tr>
<tr>
<td>Intravenous pyelogram</td>
<td>1.58</td>
</tr>
<tr>
<td>Pelvis</td>
<td>0.44</td>
</tr>
<tr>
<td>Hip</td>
<td>0.83</td>
</tr>
<tr>
<td>Extremities</td>
<td>0.01</td>
</tr>
<tr>
<td>CT scan, head or body</td>
<td>1.11</td>
</tr>
<tr>
<td>Pediatric CT scan, abdomen&lt;sup&gt;1&lt;/sup&gt;</td>
<td>25 (stomach dose)</td>
</tr>
<tr>
<td>Single screening mammogram&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3 (breast dose)</td>
</tr>
<tr>
<td>Astronaut, 3-day space shuttle mission&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2 – 3</td>
</tr>
<tr>
<td>Astronaut, 60-day space station mission&lt;sup&gt;2&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td>Average cumulative occupational dose in monitored radiation workers&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Cumulative reported badge dose 20</td>
</tr>
<tr>
<td>Average neutron-weighted colon dose for LSS population with doses between 0.005 and 4 Gy&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Colon dose 200</td>
</tr>
</tbody>
</table>


I.1.3. Radiobiology, dose fractionation and treatment planning

Radiobiology studies the effects of radiation on living organisms. Advances in radiobiology contribute substantially to understanding radiation-related cancer risk
(Preston, 2005) and to improving the efficacy of radiotherapy. For example, fractionation protocols are based on the biologic and radiobiologic characteristics of the tumour cells and of the critical normal tissues. Radiation therapy requires careful radiation treatment planning. The ultimate goal of any treatment planning system is to design a treatment that gives a high, uniform dose to the tumour while sparing normal tissue as much as possible. 3D conformed treatment planning allows one to shape radiation dose distributions around the desired tumour target, while substantially limiting the dose to nearby normal tissue. A CT scan, MRI or other modality imaging of the patient in the treatment position for tumor location is the starting point for 3-D planning (Rosenman, 2000).

I.2. Effects of radiation on DNA

I.2.1. Types of DNA damage

Many lines of evidence suggest that DNA is the target for induced cell lethality, mutation and carcinogenesis. Radiation induced DNA damage includes base lesions, DNA single strand breaks (SSB), double strand breaks (DSB), DNA-protein cross-links (CL), intrastrand, interstrand and interduplex DNA-DNA CL (Moustacchi, 2000). The yield of SSB, base and sugar lesions is one order of magnitude higher than DSB and CL. However, DSB and CL are much harder to repair, thus more lethal, cytotoxic and mutagenic (Bryant 2004; Barker et al. 2005).

I.2.2. Indirect vs direct effects

Radiation damages DNA via either indirect or direct effects (von Sonntag, 1987). The indirect effects are induced by diffusible products of water radiolysis, eg. ‘OH and eaq−. The direct effects are caused by direct ionization of DNA or by transfer of holes and
electrons to DNA from the surrounding hydration waters. In living cells both effects play a comparable role, since DNA is highly condensed in the cell and the hydroxyl radical scavenging capacity of the medium surrounding DNA is also high (Razskazovskiy et al. 2003a, b).

The indirect effects of radiation on DNA damages have been intensively studied (Chatgilialoglu and O’Neill, 2001). OH interacts with nucleobases predominantly by addition at sites of high electron density. The main base damage products by OH in cells are 8-oxoG, FapyG and thymine glycol. Other individual and tandem base lesions are also formed. OH attacks 2’-deoxyribose, resulting in a carbon-centered radical. The fate of sugar radicals depends on the environment and often leads to strand break and unaltered base release. Hydrated electrons primarily attack DNA bases, but do not result in SBs (Wala et al, 1990). 5,6-dihydropyrimidine is one of the major base lesions induced by eaq- (Falcone et al, 2005). It has been demonstrated that solvation of excess electrons in pure water occurs through two stages. The initial electron induced by ionization of water thermalizes in 110 fs (e_{pre}^-), then relaxes in 240 fs towards the full solvated state (e_{aq}^-) (Migus et al, 1987). e_{pre}^- are more reactive than e_{aq}^- toward some solutes such as SeO₄²⁻ (Pastina et al, 1999). The lifetime of e_{pre}^- is only 240 fs, three-orders of magnitude less than that of e_{aq}^-). It is unknown whether e_{pre}^- contribute to DNA damage before full solvation and whether e_{pre}^- induce the same DNA damage as e_{aq}^-). This is an interesting subject to be investigated in the future.

In contrast to the indirect effects, the direct effects of DNA damage are less well understood. The direct ionization of DNA produces holes and electrons in both bases and sugar-phosphate backbone roughly in proportion to the number of valence electrons at
each site. ESR studies show that holes mainly locate at guanine and electrons are chiefly
trapped in thymine and cytosine. ESR results also show that sugar and phosphate radicals
are produced in smaller abundance than expected from the 50% fraction of ionizations
that occur on the sugar phosphate backbone, consistent with hole transfer from the sugar
ionization sites to the bases (Cai and Sevilla, 2004). Direct radiation damage to DNA
results in a variety of products similar to those produced by the indirect effects. The
dominant products of the electron-loss centers are mainly those from guanine, such as 8-
oxo-guanine, fapy-guanine (Swarts et al. 1992; 1996) and oxazolone (Douki, 2002). The
main products of the electron-gain centers are those from cytosine and thymine, such as
5,6-dihydrothymine, 5,6-dihydrocytosine, 5,6-dihydouracil (Swarts et al. 1992; 1996;
Debije and Bernhard, 2002a, b). Sugar radicals almost invariably lead to strand breaks
(Yokoya et al. 2002; Debije and Bernhard, 2001; Debije et al. 2001) and nucleobase
release (Razskazovskiy, 2003a, b). Only recently has it been demonstrated that secondary
electrons with very low energies (even close to 0 eV) can induce DNA SSB, DSB and
base damage via dissociative electron attachment (DEA) (Boudaiffa et al. 2000a, b;
Martin et al. 2004; Dugal et al. 1999, 2000.). More details will be discussed in the next
section.

Cellular DNA contains about 20 bound water molecules per nucleotide ($\Gamma$) in the
first ($\Gamma < 9$) and second ($9 < \Gamma < 20$) layers of hydration. The first layer consists of
contiguous surface water and the second layer represents amorphous water. The direct
ionisation of water produces electrons and holes ($H_2O^+$). Electrons from both layers of
water and holes in the first hydration layer can readily transfer to DNA, but holes in the
second hydration layer react with water to form hydroxyl radicals which subsequently
attack DNA (Cai and Sevilla, 2004). The electrons and holes transferring from hydration layers to DNA modify the initial distribution and yield of DNA radicals (La Vere et al. 1996; Melian et al. 1999; DebiJE et al. 2000; DebiJE and Bernhard 2000), and influence the yields of the final DNA damage, eg. SSB, DSB, unaltered nucleobases release and base damage (Swarts et al. 1992; 1996; Yokoya et al. 2002; Falcone et al. 2005.) DebiJE and Bernhard (1999) studied free radical yields in crystalline DNA exposed to X-rays in 4 K and found that the yield strongly depends on the packing of DNA; however, it does not depend on DNA base sequence, conformation, counterion or length of base stacking. Closely packed DNA was found to trap the maximum amount of radicals, due to the minimization of the interhelical solvent space. This is consistent with inter-double strand electron tunneling (Cai and Sevilla 2000).

1.2.3. DNA damage induced by low energy electrons (LEE)

The processes induced by LEE in cells almost certainly play an important role in radiobiology. The interaction of ionizing radiation with cells triggers a series of ultra-fast events: primary, secondary and reactive (Sanche, 1989). Originating from the propagation of the initial radiation and the fast charged particles produced by the primary radiation, e.g. Compton and photoelectrons, the primary event results in the excitation and ionization of cellular constituents. The probabilities of excitation and ionization are similar, but about 80% of the deposited energy flows into ionization. Ionization leads to cations and secondary electrons (SE) (Sanche, 2002). The majority of the SE have energies below 70 eV, but they are produced in large quantities (∼10^5/MeV) (LaVerne and Pimblott, 1995). SE carry most of the energy deposited by the initial radiation, but the effects induced by SE, which occur within the femtosecond time scale, have just been
unveiled in last decade. In contrast to our precise knowledge of the radiation dose applied to patients undergoing medical diagnosis or radiotherapy, we do not have a complete picture of the biological effects induced by radiation, especially the ultra-fast processes driven by SE.

1.2.3.1 Interaction of LEE with condensed matter

LEE scattering is divided into resonant and nonresonant components (Schulz, 1973). Resonances occurring for electron impact often enhance inelastic cross sections by orders of magnitude and show a signature of isolated peaks on the energy function of these cross sections. Nonresonant cross sections generally increase slowly and smoothly with electron energy. Resonances occur when electron scatters constructively within the target and resides around the target for a much longer time than usual. From an atomic or molecular orbital view, a resonant state might be considered as a negative ion formed by an electron which temporally occupies an orbital of the target. If the incoming electron occupies a previously unfilled orbital of the target in its ground state, the transitory state is called a single particle resonance or shape resonance. If the incoming electron excites one electron of the target to a previously unfilled orbital and itself occupies another previously unfilled orbital, the transitory state is called a two-particle, one-hole state or core-excited resonance (Sanche, 1991).

According to Sanche (1991), there are six possible decay channels of the transient anion. Taking a transient DNA anion DNA\(^-\) as an example, these channels are:

1) vibrational and/or rotational excitation of DNA:

\[ \text{DNA}^- \rightarrow \text{DNA}^{*(r,v)} + e^- \]
(2) electronical / vibrational / rotational excitation of DNA

\[ \text{DNA}^- \rightarrow \text{DNA}^* (n,r,v) + e^- \]

If DNA* is dissociative, \( \text{DNA}^* (n, r, v) \rightarrow \text{SB, modified base etc.} + e^- \)

(3) (4) dissociative attachment (DA):

\[ \text{DNA}^- \rightarrow \text{SSB/DSB/MDSB/free base/CN/OCN etc.} + O' / H^- \text{ etc} \]

\[ \text{DNA}^- \rightarrow \text{SSB/DSB/MDSB/free base/CN/OCN* etc.} + O' / H^- \text{ etc} \]

(5) If certain unit of DNA, eg. thymine, has a positive electron affinity for its valence bound anion, 0.12 eV (Schiedt et. al. 1998); 0.22 eV (Li et al. 2002a)

\[ \text{DNA}^- (\text{excited state}) \rightarrow \text{DNA}^- (\text{stable, ground state}) + \text{energy to surroundings} \]

(6) If the energy of DNA^- is above the ionization potential of DNA (observed only from simple molecules in the gas phase)

\[ \text{DNA}^- \rightarrow \text{DNA}^+ + 2e^- \]

I.2.3.2 Interaction of LEE with plasmid DNA

Folkard et al (1993) were the first to study LEE effects on plasmid DNA and found threshold energies for SSB and DSB at 25 and 50 eV. DNA films on gold-coated ceramic discs were exposed to electrons of 25-4000 eV at a pressure of < 7.5 x 10^-5 torr. They used plasmid DNA mixed with EDTA and the ratio of EDTA: DNA was 1:1 (w/w). This probably resulted in the shielding of DNA from damage and likely explains why they did not see damage below 25 eV. Boudaiffa et al. (2000a) bombarded dry supercoiled plasmid DNA with electron beams of 0.1 to 1.5 keV in UHV (10^-9 torrs). DNA damage was detected by gel electrophoresis followed by quantitation of the DNA
bands by fluorescence using ethidium bromide or by hybridization with a radioactive probe. They found that electrons induce single, double and multiple double-strand breaks in supercoiled plasmid DNA.

With the second generation of UHV apparatus and the use of tantalum as a DNA substrate, Boudaiffa et al. (2000b) and Huels et al. (2004) measured the quantum yields of DNA SSB, DSB and multiple DSB (MDSB) as a function of LEE energies of 5-100 eV. Boudaiffa et al. (2000b) observed a strong peak (~ 8 x 10^{-4} SSB and ~ 2 x 10^{-4} DSB per incident electron) around 10 eV for both SSB and DSB yield functions, and a very weak peak (~ 1.5 x 10^{-5} MDSB per incident electron) at 25 eV for MDSB. Boudaiffa et al. (2000b) compared their resonance structures with peaks in the yields of the induction of the anion and neutral fragments by LEE in primary constituents of DNA (e.g. bases, sugar, phosphate and structural H₂O) as a function of electron energy. They concluded that the maxima around 10 eV resulted from the formation of transient anions, decaying into the DEA and/or dissociative electronic excitation channels. However, since all the basic DNA units can be broken via DEA between 3-13 eV, it is impossible to attribute SSB and DSB to the initial dissociation of a specific component. Above 15 eV the yields of SSB, DSB and MDSB rise monotonically as a function of electron energy. Nonresonant mechanisms related to excitation/ionizations/dissociations contribute dominantly to the above yields. Boudaiffa et al (2002) also derived the total effective cross section (3.4 x 10^{-15}, 3.8 x 10^{-15}, 4.4 x 10^{-15} cm²) and effective range (12, 14, 14 nm) for the destruction of supercoiled plasmid DNA at 10, 30 and 50 eV, respectively.

With the development of more sensitive techniques to detect DNA SSB and DSB using Sybr® Green I, Martin et al. (2004) extended the study of SSB and DSB in plasmid
DNA by LEE to the lower energy range of 0.1-5 eV. Small electron beam currents (2nA) and exposure times (≤ 20 s) were used in their study to avoid film charging and thus beam defocusing. The quantum yields of DNA SSB in plasmid DNA as a function of LEE energies showed two peaks (~ 1 × 10⁻² and ~ 7.5 × 10⁻³ SSB per incident electron) at incident electron energies of 0.8 and 2.2 eV. It was proposed that the formation of a transient anion occurs via electron capture in the π⁺ anion state of the bases, followed by charge transfer to a σ⁺ anion state of the phosphate group and the rupture of C-O bond connecting the phosphate group to the sugar (Barrios et al. 2002).

Pan et al (2003) measured electron stimulated desorption (ESD) of H⁻, O⁻ and OH⁻ from plasmid DNA within the 3-20 eV range and observed resonant structure with a maxima around 9 eV. They demonstrated that below 15 eV LEE-induced H⁻ desorption from DNA occurs via DEA to the bases with a substantial contribution from the deoxyribose ring, and O⁻ desorption via DEA to the phosphate group as predicted by Li et al (2003). They proposed that the OH⁻ desorption resulted from a two step process: formation of O⁻ via DEA to the phosphate group followed by reactive scattering of the O⁻ ion with the nearby deoxyribose unit.

I.2.3.3 Interaction of LEE with oligonucleotides

Thiolated oligonucleotides may be prepared as self-assembled monolayers (SAM) chemisorbed on gold via S-Au bonding (Nogues et al. 2004). Such samples have the advantage of being more uniform in coverage, better oriented and purer than those made from plasmid DNA. Dugal et al. (1999, 2000) and Abdoul-Carime et al. (2000a, 2000b, 2001, 2002) measured the yields of neutral fragments induced by 1-30 eV electrons that
impinged on SAMs of 8 oligonucleotides (CCCCCC; CCCCCCCC; CCCCCCCCC; TTTTTT; TTTTTTTTTT; TTTTTTTCT; CCCCCCTTTT). Their results were obtained from mass spectrometric measurements of the residual atmosphere near the target during its bombardment in UHV by a 10 nA electron beam. The desorption of CN*, OCN*, and/or H2NCN neutral species had the most intense observable yields; however, no sugar moiety, phosphorus-containing fragments or entire nucleobases were detected. The total fragment yields of CCCCCCCCCCCC, 5'-TTTTTTCCC-3' and TTTTTTTTTTT at 12 eV were found to be 5.8, 5.0 and 3.9 x 10^3 fragment/electron, respectively (Abdoul-Carime and Sanche 2001). These yields are ~ 5 times as much as the yield of SSB observed at the same incident electron energy, suggesting more base damage than SSB. The total effective cross section (σ_eff) per base estimated for the CN*, OCN* production from fragmentation of CCCCCCCCCCCC, CCCCCCTTT, TTTTTCCC and TTTTTTTTTTTT at 12 eV incident electron energy were (3.4, 2.0, 2.9 and 2.3) x 10^{-17} cm^2, respectively (Abdoul-Carime and Sanche 2002). The incident electron energy dependence of σ_eff for desorption of these fragments exhibited structures at electron energy lower than 20 eV, which are characteristic of transient anion formation. They concluded that at incident electron energies <20 eV, neutral fragment desorption arises from dissociation of the DNA bases, principally via DEA and/or decay of the transient anion into a dissociative electronic excited state of the bases. Non-resonant mechanisms (e.g. direct dipolar dissociation) mostly control the fragmentation processes at electron energy higher than 20 eV. From comparison of the electron energy dependence of the σ_eff for base
fragmentation in the homo- and heteronucleotides, they further concluded that damage to a short DNA strand is dependent on base identity, sequence and electron energy.

Pan and Sanche (2005) studied ESD of OH$^-$ from SAM of both single and double stranded 40-mer oligonucleotides chemisorbed on a gold substrate either lying flat or standing up on the substrate. The counter ions of their DNA were purposely replaced with H$^+$. They found between 2 and 5 eV, OH$^-$ desorption occurred exclusively via direct DEA to the phosphate unit. Above 5 eV, direct DEA was mainly responsible for the OH$^-$ induction; however there is also the possibility of a contribution from reactive scattering of O$^-$. They proposed that the phosphate-counterion part of DNA plays a significant role in LEE induced DNA damage.

Ray et al. (2005) studied temporary trapping of LEE (<2 eV) by SAM of 15-mer oligonucleotides chemisorbed on gold and two-photon electron ejection from the same DNA. In their experiment, photoelectrons are either ejected by an excimer laser operating at 193 nm (6.4 eV) from a gold substrate, or are excited with photon energy (3.55 eV) below the work function of gold (~5 eV). For the former, the instantaneous transmitted current through DNA as a function of electron energy is measured using a time of flight spectrometer. This current reflects the LEE capturing efficiency of the DNA layer. In the later case, some of the excited electrons are assumed to be transferred to the LUMO of oligonucleotides. A second photon (3.55 eV) is used to eject these electrons from the LUMO to the vacuum. The kinetic energy of ejected electrons, which is related to their binding energy, is measured. However, it appears that more experimental evidence is needed to distinguish whether the recorded electron spectra are really from two-photon photoelectrons or from two-photon excitation. Ray et al. suggest
that: (1) the electron capturing probability depends on the number and clustering of guanine and strandness of DNA; (2) the captured electron is lying either on the sugar phosphate backbone or between the molecules in the layer in a nonlocalized state; (3) the state of the captured electrons is insensitive to the sequence of the DNA and (4) the electrons are more strongly bound in the double than in the single stranded DNA. Electron localization on the sugar-phosphate backbone is consistent with electron transfer from the bases leading to DNA damage (Martin et al 2004, Barrios et al 2002).

I.3. X-ray induced electron emission from metal

Exposure of metal to X-rays results in a large amount of backscattering secondary electrons (SE) from metal surface. The energy spectra of the emitted electrons typically exhibit a narrow peak below 100 eV for the low energy part and relatively sharper elastically scattered photoelectron and Auger electron peaks at high energy (Henke et al. 1981). The quantum yields for fast and slow SE (\(\eta_f\) and \(\eta_s\)) vary approximately as \(E^2\mu_{en}(E)/\rho\) and \(E\mu_{en}(E)/\rho\), respectively. For slow SE (\(E_k \leq 100\) eV), the energy distribution can be described with equation 1 (Henke et al 1977):

\[
\eta(E_k) = \eta_s E_k^4 / (E_k + W)^4
\]

(1)

where \(\eta(E_k)\) and \(\eta_s\) are the yields of SE having kinetic energy of \(E_k\) and 0-100 eV, respectively; \(W\) is the work function of the metal. The shapes of these distributions do not depend significantly upon photon energy.

Radiation dose perturbation at low and high Z material interfaces has long been a subject of investigation in the fields of medical physics and radiation oncology. The dose enhancement at the tissue-metal interface has been confirmed by dose measurements
(Niroomand-rad et al. 1996; Allal et al. 1998; Melian et al. 1999) and Monte Carlo calculations (Li et al. 1999, 2002b; Verhaegen and Seuntjens, 1995). Niroomand-rad et al (1996) reported a dose enhancement of about 20% at the upper surface of the tissue-titanium dental implant interface in head and neck cancer patients treated with 6 MV and 10 MV photon beams. Head and neck cancer patients having surgery to remove tumors located in the oropharynx and oral cavity often require mandible reconstruction. In addition, these patients usually need postoperative radiotherapy. Thermoluminescent dosimetry measurements by Allal et al (1998) showed marginally significant dose enhancement (6 ± 5%) at bone/titanium interfaces around a titanium hollow-screw osseointegrating reconstruction plate (THORP) using both $^{60}$Co and 6 MV photon beams. Melian et al (1999) reported a higher dose enhancement percentage, eg. 40% at the Vitallium plate using $^{60}$Co. The enhancement factor was found to increase with the effective atomic number of the plate. Li et al (2002b) reported a 5-10% overdose at a depth of 0.5 mm from the metallic esophageal stents for 6 MV, 15 MV external photon beams and for a $^{192}$Ir brachytherapy source.

The corresponding enhanced biological effects at the interfaces of tissue and metals are also documented in the literature. Regulla et al (1998) reported dose enhancements up to more than a factor of 100 in tissue-equivalent polymethylmethacrylate close to a thin metallic gold foil using 40 to 120 kV heavily filtered X-rays. Correspondingly, enhanced interface biological effects were observed, such as cell inactivation (Regulla et al., 1998), the induction of dicentric chromosomes, centric rings and excess acentric fragments (Regulla et al., 2001), as well as relative biological effects for cytogenetic effects (Regulla et al., 2002). X-ray induced SE
emission from the gold surface was proposed to account for this interface enhancement effect. Zellmer et al (1998) and Rosengren et al (1993) also reported enhanced cell inactivation due to backscattered SE from metal surface induced by X-rays and γ-rays. An investigation of the DNA damage induced by backscattered SE might provide insights into the molecular basis for the observed biological effects; however, such a study has never been reported.

I.4. Research Project

X-rays are widely used in diagnostic medical examinations and radiotherapy. The interaction of X-rays with cells produces large amounts of SE with the most probable energy lying below 70 eV. The interaction of X-rays with high Z materials such as metal implants and contrast media also induces large amounts of back-scattering SE. The energy spectrum of these SE typically exhibits a narrow peak below 10 eV for the low energy part and relatively sharper elastically scattered photoelectron and Auger electron peaks at high energy. The relative efficiency of DNA damage induced by X-ray photons and SE is important in radiobiology, is useful to understand and predict DNA damage induced by radiation during medical examination and radiotherapy, to simulate DNA damage in cells via Monte Carlo calculations, and in radiotherapy treatment planning. Attenuation length (AL) for scattering of LEE within DNA and the effective cross sections for LEE- induced DNA damage are of considerable importance to radiation biology, and are also necessary parameters for predicting DNA damage in cells induced by ionizing radiation using Monte Carlo simulations. However, such knowledge is not available in the literature. Therefore, this research project included three objectives. 1) to compare DNA damage induced by X-ray photons and low energy SE in vacuum; 2) to
examine the enhanced DNA damage by soft X-ray induced backscattering SE in air; and 3) to measure AL for scattering of LEE within DNA and the effective cross sections for LEE-induced DNA SB. Objective 1 resulted in three manuscripts:

Article No. 1: Dosimetry of ultrasoft X-rays (1.5 keV Alkα): using radiochromatic films and color scanners

Article No. 2: DNA inter-duplex crosslinks induced by Alkα X-rays under vacuum

Article No. 3: Comparison between X-ray photon and secondary electron damage to DNA in vacuum

Article No. 1 concerns the development of a dosimetry method to measure the absorbed dose of Alkα X-rays (1.5 keV) in UHV using radiochromatic dosimetry films and a color scanner. This provides the value for the absorbed dose in dry biological samples irradiated under UHV, allowing direct comparison of DNA damage induced by Alkα X-rays and SE.

Article No. 2 is to demonstrate the formation of DNA inter-duplex crosslinks by Alkα X-rays under vacuum. This is in fact a byproduct of Objective 1, since the formation of DNA inter-duplex crosslinks by Alkα X-rays was a surprising discovery.

Objective 2 resulted in Article No. 4: Enhanced DNA by secondary electron emission from a tantalum surface exposed to clinical X-rays.

Objective 3 resulted in Article No. 5: Induction of strand breaks by low energy electrons (8-68 eV) in a self assembled monolayer of oligonucleotides: effective cross sections and attenuation lengths.
II. RESULTS

Dosimetry of Ultrasoft X-rays (1.5 keV AlKα) Using Radiochromatic Films and Color Scanners

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Short running title: Dosimetry of ultrasoft X-rays

Classification numbers (PACS):
87.53.Dq Photon dosimetry: measurements
87.66.Cd Films: radiochromic etc.
II. RESULTS

Abstract This work explores the possibility of measuring the absorbed dose of ultrasoft X-rays (USX, 1.5 keV AlKα) with the GAFCHROMIC® HD-810 radiochromatic dosimetry films (HD-810 films) and color scanners. HD-810 films were exposed to USX, soft X-rays (14.8 keV) and γ-rays (60Co) for various times. The response of HD-810 films to absorbed doses of γ-rays in water was calibrated with Fricke dosimetry and used for the calibration of USX. The optical density of the HD-810 films was quantified with a HP ScanJet 6100C scanner and Corel Picture Paint 7. The choice of the reading channel and color adjustment settings were optimized to either improve sensitivity or expand the measurable dose range. The response of the HD-810 films to the absorbed dose in water decreased by 50 % when the effective photon energy decreased from 1.25 MeV to 14.8 keV. The ratio of the mass energy absorption coefficient of the active layer of HD-810 films to that of water was found to play a major role in this decrease. The mean absorbed doses of the active layer of the HD-810 films exposed to USX were derived. The calculation of the initial photon fluence rate and the mean absorbed doses of USX to biological samples such as plasmid DNA is discussed. This study suggests that radiochromatic dosimetry films are promising secondary dosimeters for measuring the absorbed dose of USX.
Introduction

Ultrasoft X-rays (USX, 0.28 keV to 4.55 keV) have been actively used in biological, biochemistry and radiation chemical investigations for several decades because they interact with material mainly via the photoelectric effect and produce very short and isolated tracks of electrons with well-defined energies (Fulford et al 1995, Hill et al 1998, Goodhead et al 1981, Bryant et al 2003, de Lara et al 2001). An air filled ionization chamber is usually used for dosimetry of USX (de Lara et al 2001). Ionization chambers are primary dosimeters, often used as calibration for secondary dosimeters; however, ionization chambers usually are not practically convenient. In fact, due to their short penetration in matter (for 1.5 keV X-rays, the intensity is reduced 50 % by 4.8 mm of air) (Hubbell and Seltzer 1995), USX irradiation requires special attention to minimize the attenuation of the X-rays between the X-ray source and the samples (Klyachko et al 1999, Fulford et al 1995, Hill et al 1998). One solution is to perform the USX irradiation under vacuum (Klyachko et al., 1999). In such an environment, dosimetry is difficult if not impossible with conventional equipment. It would be useful to find a simple alternative dosimeter for USX. Several radiation detectors such as radiographic film, radiochromic film, Scanditronix semiconductors, thermoluminescence dosimeters (TLDs) and metal oxide semiconductor field effect transistors (MOSFETs) are among the possible candidates (Edwards et al 1997, Kron et al 1998). Radiochromic film, which appears to be the easiest to use, especially in a vacuum chamber where sample introduction is difficult, was therefore chosen for this study.

GAFCHROMIC® radiochromatic dosimetry films (GAF films, the Advanced Materials Group of International Specialty Products) are self-developing film media,
designed for the measurement of absorbed doses of high-energy photons and electrons and have been widely applied in the mapping of dose distributions from clinical radiation sources since the early 1990s (Bazioglou and Kalef-Ezra 2001, Buenfil et al 2002, Butson et al 2000, Li et al 2000, McLaughlin et al 1991, Muench et al 1991, Odero et al 2001, Schumer et al 1999, Stevens et al 1996). Their responses are reported to be dose-rate independent and energy independent down to about 0.2 MeV (McLaughlin et al 1991, Muench et al 1991, Li et al 2000). Muench et al (1991) reported that their response decreases by about 30% when the effective photon energy decreases from 1710 keV (4 MV x-rays) to 28 keV (60k Vp x-rays, 2-mm Al filter). Recently Kron et al (1998) studied the variation of dose response with photon energy down to 10 keV and found significant decreases of response by about 50% at effective energies of 21-26 keV, depending on the type of the GAF films. However, no attempt to use GAF films for photon energies lower than 10 keV has been reported.

Unexposed GAF films are transparent and upon exposure to X- or γ-rays the blue color of the film develops in proportion to the absorbed dose. Several studies have used document scanners to quantify the optical density of GAF films (Bazioglou and Kalef-Ezra 2001, Buenfil et al 2002, Stevens et al 1996). Surprisingly, "Grayscale" output was chosen and suggested to be superior to "True Color" output. In fact, the advantages of using True Color have not been determined and reported.

This paper reports the first attempt to measure the absorbed dose of USX (Al_{kα} X-rays, 1.5 keV) with GAFCHROMIC® HD-810 radiochromatic dosimetry films (HD-810 films). The responses of the HD-810 films with exposure time to USX, soft X-rays and γ-rays are studied. The dose responses were quantified with a HP ScanJet 6100C.
scanner and Corel Picture-Paint 7. The effects of channel choice and color adjustment on the dose response is also studied. From these experiments, we suggest scanning settings for various ranges of absorbed doses. This work shows that radiochromatic dosimetry films are a promising secondary dosimeter for USX.

Experiments

**Dosimetry film**

GAFCHROMIC® HD-810 radiochromatic dosimetry films were generously provided by Dr. David F. Lewis (Advanced Materials Group, International Specialty Products(ISP), NJ, USA). The film is composed of three layers: a gelatin surface layer (0.75 μm); an active layer (6.5 μm) and a transparent polyester support layer (97 μm). The density and composition of surface and active layers are shown in Table 1.

**Irradiation**

γ-ray irradiation was performed with a $^{60}$Co Gammacell 220 (Atomic Energy of Canada Limited). The absorbed dose rate to water was determined by Fricke dosimetry as 3.95 Gy min$^{-1}$ at the center of the irradiation chamber. 400 μL of Fricke solution was irradiated in a 0.5 mL Self-Lock Eppendorf tube, held in a plastic rack. A piece of HD-810 film (0.5 cm × 1.0 cm) was placed on a 1 mm thick plastic support at the center of the irradiation chamber and irradiated for various times from seconds to hours. The $^{60}$Co sources were vertically aligned rods around the circumference of the chamber, thus, the γ-rays came from the circumference of the chamber and the full build-up was achieved at
the position of the sensitive layer of HD-810 film. The impact of the different scattering conditions on the relative dose to the film and the Fricke solution was considered to be negligible.

Soft X-rays were generated by the superficial X-ray unit (PANTAK Therapax 3 series) at the University of Sherbrooke Hospital Center, Sherbrooke, Quebec, Canada. A 0.4 mm Al filter, a potential energy of 30 kVp and a filament current of 7.6 mA were selected, resulting in an X-ray beam with an effective energy of 14.8 keV, determined by the half-value layer (HVL). A treatment cylinder (3.0 cm diameter, 15 cm source surface distance (SSD)) was used to expose a sample of HD-810 film (0.5 cm × 1.0 cm) to X-rays. The film was placed on a block of tissue equivalent plastic at SSD of 15 cm and covered by the treatment cylinder. The absorbed dose rate at the surface of the treatment cylinder was determined with an NBS traceable standard Victoreen r-meter to be 2.41 Gy min⁻¹ (Coffey et al 1989).

Al_kα X-rays were produced using a PHI Model 04-548 Dual Anode X-ray Source (Perkin Elmer) with an aluminum target. The characteristic X-ray (Al_kα) energy is 1486.6 eV with FWHM of 0.85 eV. The X-ray emission spectrum exhibits not only the characteristic X-rays, but some minor x-ray components at higher photon energies (X-ray satellites). The energies and relative intensities (compared to Al_kα; 1486.6 eV) of the X-ray satellites are: 1496.4 eV (6.4 %), 1498.4 eV (3.2 %), 1506.7 eV (0.4 %), 1510 eV (0.3 %), 1556 eV (0.55 %), respectively. Therefore, the USX energy was taken as 1.5 keV in this study. The HD-810 film (1.8 cm × 1.0 cm) was fixed on a stainless steel target, irradiated by Al_k X-rays in an ultra-high vacuum (UHV) chamber at pressures of 3
x $10^{-9}$ torrs for various periods of time. Details of the apparatus have been described elsewhere (Klyachko et al 1999). The fluence of photons was adjusted by the nominal power applied to the anode with the voltage kept constant at 4 kV, at which the percentage of Bremmstrahlung radiation relative to Al$_{\alpha}$ X-rays has been reported to be less than 1% (Hoshi et al., 1985). The percentage of Bremmstrahlung radiation increases with the voltage such that we found that at 15 kV 80% of absorbed dose in HD-810 film was from Bremmstrahlung radiation. Thus it is important to use the lowest operable voltage to minimize the Bremmstrahlung radiation. A 2 μm thick aluminum foil window was used to further minimize the Bremmstrahlung background and to block the heat and light from the X ray source. Four nominal power levels (8, 20, 35 and 80 Watts) were selected in this study with measured emission currents of 1.1, 3.8, 7.2 and 18.1 mA, respectively.

*Measurement of the optical densities of the HD-810 films*

HD-810 films were stored for over 48 hours after irradiation at room temperature in the dark before measurement of the optical density. The density grows rapidly with time immediately after exposure but stabilizes within about 24 hours (Buenfil Li et al 2002, Li et al 2000). This was confirmed by a time dependent study in this work. The films were scanned using a HP ScanJet 6100C color scanner, which was driven by HP Precision Scan Pro 1.01. Auto exposure and sharpen level were turned off. The intelligent scanning technology for best quality scaling, best quality sharpening, maximum pixel depth and best quality scan speed was also turned off from the Preferences menu. It is very important to turn off the above settings to ensure the reproducibility of scans. The output resolution was set at 150 dpi. The output type was set
as “true color”, except for comparison when “grayscale” was used. The Color Adjustment tool can be used to change the color balance (hue) and color saturation of the selection area. The amount of a particular color (hue) in a color image can be increased or decreased by changing the arbitrary values between -100 to 100 for the X and Y coordinates. The overall intensity (saturation) of the color in an image can be adjusted between 0 % to 150 %. In this study, the color saturation was kept constant at 100 %. The color hue was set as desired. Five color hues (x = 0, y = 0 for blank; x = 100, y = 0 for red; x = -100, y = 0 for blue; x = 0, y = 100 for green; x = 0, y = -100 for violet) were tested in this study. The scanned images were processed with Corel Picture-Paint 7. The average pixels (S) for RGB, Red (R), Green (G), Blue (B) or Gray channels were read from the histogram, respectively.

The net optical density (absorbance, ABS) was defined as log$_{10}$(S$_0$/S) (Stevens et al 1996), where S$_0$ is the average pixel of unexposed films. The response of ABS to the absorbed dose was described by equations 1 (Odero et al 2001) or 2 (Kron et al 1998):

For a restricted data set at the lower doses comprising the linear part of the response curve and readout from all the channels,

$$\text{ABS} = A \times \text{Dose} \tag{1}$$

For a full data set covering the response curve up to the saturation and readout from the RGB, G or B channels,

$$\text{ABS} = B \times [1 - \exp (-A \times \text{Dose})] \tag{2}$$

A and B are constants. Equation 2 cannot apply to the readout from R channel since its response curve behaves differently from the other channels. Beyond the linear
region the ABS rises rapidly to a sharp peak then gradually falls. This behavior cannot be described by a simple equation.

Typically 25 pieces of $1.0 \times 1.8$ cm HD-180 film (about $800 \times 800$ pixels) were processed per scan. Since the dose over the exposed area is not completely uniform, only the center portion of the film was analyzed ($1 \times 1$ mm; about 100 pixels).

All manipulations were performed in low lighting.

Results

1. The effect of color adjustments and channel selection on dose response curves for $\gamma$-rays

Both Bazioglou and Kalef-Ezra (2001) and Stevens et al (1996) used document scanners to measure the ABS of GAF dosimetry film and chose the “grayscale” output, mentioning the smoother dose response with “grayscale” than “true color”. The “grayscale” and “true color” outputs were compared in this work. When the color adjustment was turned off and the readout was from the RGB channel, the “true color” output was found to be slightly more sensitive than the “grayscale”, while the smoothness of the dose response was similar for both output types. However, the “true color” output permits both color adjustments and reading on three color channels. The effects of color adjustment and channel choice on the dose response curves were studied in detail by irradiating HD-810 films with $\gamma$-rays. The absorbed doses of $\gamma$-rays to water ($\gamma D_{water}$) was measured by Fricke dosimetry.

Table 2 compares the sensitivity, the standard deviation (SD) of pixels in a scanned image as well as the resulted SD of ABS, measurable dose ranges and regression
fits of ABS to $^7$D$_{\text{water}}$ at the optimal color adjustment setting for each channel. Both the channel choice and the color adjustment setting significantly affected the sensitivity, but had little effect on the SD of pixels. However, the effect of the former parameter on the measurable dose range was more significant than that of the latter. The choice of Red channel resulted in the highest sensitivity, while the choice of Blue channel led to the highest measurable dose. The calibration curves of ABS vs. $^7$D$_{\text{water}}$ obtained from the Red channel and the Blue channel at the optimal color adjustment setting are shown in Figures 1 and 2, respectively. The error bars show the SDs of ABS resulting from the SDs of pixels in the both scanned non-irradiated and irradiated images.

The relative sample-to-sample variability in the measured ABS for a given dose is about 3 %. The absolute ABS variability is dose dependent, but the relative error is dose independent. The reproducibility of the scanner from different measurements of ABS done on the same film at different times (but 24 hours after irradiation) or different positions on the scanner is comparable to the sample-to-sample reproducibility, with a relative variability of less than 4 %.

2. Measuring the absorbed dose of soft X-rays with an effective energy of 14.8 keV

HD-810 films were exposed to soft X-rays with an effective energy of 14.8 keV for various times up to 25 minutes. The absorbed dose rate to water was determined with an NBS traceable standard Victoreen r-meter to be 2.41Gy min$^{-1}$ (Coffey et al 1989). The ABS of exposed films was read from the R channel with the color adjustment set at $x = -100$, $y = 0$. Figure 1 shows the dependence of ABS on the absorbed dose of soft X-rays to
water. The linear regression fit of experimental data to equation 1 gave $A = (5.01 \pm 0.06) \times 10^3 \text{ Gy}^{-1}$ and $R^2 = 0.9998$, respectively.

3. *Measuring the absorbed dose of ultrasoft X-rays*

HD-810 films were exposed to Al$_{\text{K}\alpha}$ X-rays for various times with the anode at a constant voltage of 4 kV and four emission currents of 1.1, 3.8, 7.2 and 18.1 mA, respectively. The ABS of the exposed films was read from the B channel with the color adjustment set at $x = 0$, $y = 100$. Figure 3 shows the dependence of ABS measured at the center of the target on the irradiation time as a function of the measured emission currents. The ABS increased both with exposure time and emission current.

The Al$_{\text{K}\alpha}$ line (1.5 keV) is substantially different from the K shell edges for H (13.6 eV), C (284.2 eV), N (409.9 eV) and O (543.1 eV) (Henke *et al* 1993), the elements present in the active layer of HD-810 films. In addition, the solid-state polymerization reaction in the active layer is a simple first-order process (McLaughlin *et al* 1994). Thus, the formation of the blue polymer product in the HD-810 films is assumed to be characterized by an average energy deposition independent of radiation quality (Prestwich and Murphy 2000). The reliability of this assumption will be examined and discussed in section 2 of the Discussion. Therefore the mean absorbed dose of Al$_{\text{K}\alpha}$ USX to the active layer (BL) of HD-810 film ($^{\text{USX}}D_{\text{BL,m}}$) can be derived from the calibration curve of Fig. 2 using equations 3 and 4:

$$ ^\gamma D_{\text{water}} = -1/0.62 \times \ln(1 - \text{ABS/1.24}) $$

$$ ^{\text{USX}}D_{\text{BL,m}} = ^\gamma D_{\text{BL}} = ^\gamma D_{\text{water}} \times ^\gamma \mu_{\text{BL}} / ^\gamma \mu_{\text{water}} \cong ^\gamma D_{\text{water}} $$

30
where the $\mu$'s are the mass energy absorption coefficients calculated from weight composition and mass energy absorption coefficients of each element (Table 3) (Hubbell and Seltzer 1995).

Figure 4 shows that the $U_{\alpha}X D_{BL,m}$ increased linearly with the exposure time. The linear regression fit to the experimental data gave the following mean absorbed dose rates (slope in kGy s${}^{-1}$) and $R^2$s to the active layer of HD-810 film of: $(1.15 \pm 0.02) \times 10^{-3} \text{ and } 0.998; (5.0 \pm 0.1) \times 10^{-3} \text{ and } 0.990, (9.7 \pm 0.3) \times 10^{-3} \text{ and } 0.987; (26.1 \pm 0.4) \times 10^{-3} \text{ and } 0.998$ for emission currents of 1.1, 3.8, 7.2 and 18.1 mA, respectively. Fig. 4a shows that the mean absorbed dose rate increased linearly with emission currents. The linear regression fit to the experimental data gave a slope of $(1.42 \pm 0.02) \text{ Gy s}^{-1} \text{ mA}^{-1}$ and $R^2 = 0.9998$.

4. **Effect of the water content of HD-810 films on the response to radiation**

Upon being desiccated in the UHV chamber ($3 \times 10^{-9}$ torr) for 48 hours, HD-810 films were exposed to $\gamma$-rays for 5, 10, 15 and 20 seconds. The response of the desiccated HD-810 films was found to be the same as that of ordinary HD-810 films (containing 7.5 % water in the active layer). This result suggests that the water content has no effect on the response of HD-810 to radiation, in good agreement with the results of Janovsky and Mehta (1994).

5. **The attenuation of USX by HD-810 films**

Three sheets of HD-810 film were overlapped and exposed to USX at a nominal power level of 80 Watts for 100 seconds in the UHV chamber. The ABS of both lower sheets of HD-810 film was below the detection limit. This result is in agreement with our
II. RESULTS

calculation of the attenuation of photon intensity by HD-810 film using the mass attenuation coefficients (see Table 3) (Hubbell and Seltzer 1995) and argues that there is very little Bremsstrahlung contamination in the Al_{ka} X-rays used in this study.

Discussion

1. Using color scanners in the readout of optical density of GAF films

When the active component in GAF films is exposed to radiation, it reacts to form a blue colored polymer with absorption maxima at about 615 nm and 675 nm (Li et al 2000, McLaughlin et al 1991, Muench et al 1991). Several methods can be used to determine the optical density of GAF films, including spectrophotometers, densitometers and flatbed color scanners; however, only densitometers and color scanners are suitable for quantification of the spots.

Since the response of GAF films is enhanced by measurement at the peak absorbance, spectrophotometers have an advantage over densitometers and color scanners in that they can be used either to maximize the sensitivity by measuring at 675 nm or maximize the dose range (up to 50 kGy) by using a non-peak wavelength (Li et al 2000). Most densitometers employ an optimum red LED light source and filter to maximize the response, thus reducing both the minimum and maximum limits for the measurable absorbed doses.

Unlike densitometers and spectrophotometers, color scanners are more commonly available and usually do not need any modification for the measurement of ABS. Although several studies have used document scanners for ABS measurement of GAF
films, surprisingly, only the “grayscale” output type was chosen (Bazioglou and Kalef-Ezra 2001, Buenfil et al 2002, Stevens et al 1996). These studies led to a false impression that scanners are less than ideal substitutes for densitometers since they are somewhat inferior with respect to sensitivity and linearity of response.

Our results with a color scanner show that the “true color” output type is a better choice than “grayscale”. The reading channel functions as a filter and limits the measurement to the three absorption ranges of Red, Green and Blue. Readout of the R channel resulted in the highest sensitivity and readout of the B channel decreased the sensitivity but increased the measurable absorbed doses up to 5.5 kGy. In addition, the color adjustment increases the scanning light for certain colors, thus increasing the response.

Readout from the R channel with the color adjustment set at $x = -100, y = 0$ gave a sensitivity of $(1.00 \pm 0.04) \times 10^{-2}$ ABS Gy$^{-1}$ within the $\gamma$-absorbed dose range of 0 – 100 Gy for HD-810 films. This sensitivity is slightly higher than the $8 \times 10^{-3}$ ABS Gy$^{-1}$ obtained with the Nuclear Associate Radiochromic Densitometer Model 37-443, recommended by the ISP (2002).

In conclusion, with the appropriate choice of channel and color adjustment, color scanners are superior to densitometers with respect to economy, sensitivity and measurable dose ranges and to spectrophotometers with respect to spot measurement.

2. *The effect of photon energy on the response of GAF films to the absorbed dose in water and the role of mass energy absorption coefficients*
The response of GAF films to the absorbed dose in water is energy-independent for photons above about 0.2 MeV, at which energies they are tissue or water equivalent (Butson et al 2000, Li et al 2000, McLaughlin et al 1991). Muench et al (1991) reported that the response decreases by about 30 % when the effective photon energy decreases from 1710 keV to 28 keV. Kron et al (1998) found significant decreases of response, about 50 %, at effective photon energies of 21-26 keV. The ratio of mass energy absorption coefficients between the active layer of GAF films and water was found to play a major role in the variation of the dose response with effective photon energy. Other factors, such as the mixture of different materials in the film and the dependence of G (Blue Polymer) on photon energy might play minor roles.

In the present study, we found that the response of HD-810 films to the dose absorbed in water decreased by 50 % when the effective photon energy decreased from 1.25 MeV to 14.8 keV in the fair agreement with the results of Kron et al (1998). Table 3 shows mass energy absorption coefficients of water, each layer of HD-810 film as well as the attenuation of photon intensity (I/I₀) at three levels of photon energy. For each layer of HD-810 films, μₑₑ/ρ was obtained by simple additivity using μₑₑ/ρ = Σ fᵢ (μₑₑ/ρ)ᵢ (Hubbell and Seltzer 1995) and ignoring the radiative losses (bremsstrahlung production, annihilation in flight, etc.). The uncertainty of the weight composition (f), mass energy absorption coefficients (μₑₑ/ρ) and simple additivity all contribute to the accuracy of the calculation of the expected decrease in response, the ratio of (μₑₑ/ρ)ₓ to (μₑₑ/ρ)ₜₐₚ₉ water for 14.8 keV soft X-rays. The accuracy is thus estimated to be about 90 %. The ratio of (μₑₑ/ρ)ₓ to (μₑₑ/ρ)ₜₐₚ₉ water at a photon energy of 14.8 keV and 1.25 MeV is 0.55 and 0.98, respectively, predicting the decrease in response by 38-49 %. In addition, there are
uncertainties of 1% and 4% involved in the experimental measurement of the response of HD-810 films to soft X-rays and γ-rays, respectively. Therefore the decrease in the ratio of \((\mu_{\text{en}}/\rho)_{\text{BL}}\) to \((\mu_{\text{en}}/\rho)_{\text{water}}\) at 14.8 keV compared to the ratio at 1.25 MeV can account for the 50% decrease in the response of HD-810 films to soft X-rays versus γ-rays observed in this work. This result supports the assumption that the formation of the blue polymer product in the HD-810 film is characterized by an average energy deposition independent of radiation quality, as assumed by Prestwich and Murphy (2000) and in this work.

Ideally, the response of the HD-810 film to USX should be compared directly with an alternative primary method of dosimetry such as an ionization chamber or a gas flow proportional counter (Hoshi et al., 1985). Unfortunately, many Alkα X-ray sources, including the one used in this study, can only be operated under vacuum (< 10^-7 torr), while both ionization chambers and gas flow proportional counters must be used at ambient pressure. Thus, this study demonstrates the utility of HD-810 films as secondary dosimeters for measuring the absorbed dose of USX. Further studies on comparing the response of the HD-810 film to USX directly with a primary dosimetry method will be necessary for accurate measurement of the absolute dose for USX.

3. Estimate of the initial photon fluence rate of USX and the mean absorbed dose in dry biological samples

For photon energies as low as 1.5 keV, the photon intensity can be dramatically attenuated by the sample. As shown in Table 2, over 99.9% of γ-rays and 14.8 keV photons pass both the surface and active layers of HD-810 films. However, for 1.5 keV
USX, 7% of the photon fluence was attenuated by the surface layer and 41% by the active layer in vacuum. Thus, the measured absorbed dose rate shown in Fig. 4 is the mean absorbed dose rate to the active layer (BL) of HD-810 films. The surface dose rate of the active layer could be calculated using (Fulford et al 1999):

\[
\text{USX} \cdot D_S = \text{USX} \cdot D_m \times (\mu_{en}/\rho \times \rho \times d) / [1 - \exp(-\mu_{en}/\rho \times \rho \times d)]
\]

where \( \mu_{en}/\rho \) is the mass energy absorption coefficient in \( \text{cm}^2 \text{g}^{-1} \), \( \rho \) is the density in \( \text{g cm}^{-3} \) and \( d \) is the thickness in cm. For the active layer in vacuum, \( \mu_{en}/\rho = 809 \text{ cm}^2 \text{g}^{-1} \), \( \rho = 1.0 \text{ g cm}^{-3} \) and \( d = 6.5 \times 10^{-4} \text{ cm} \), resulting in \( \text{USX} \cdot D_{BL,S} = 1.29 \text{USX} \cdot D_{BL,m} \).

The photon fluence rate (\( \phi_{BL} \) in photons \( \text{s}^{-1} \text{cm}^{-2} \)) at the incident surface of the active layer can be calculated from \( \text{USX} \cdot D_{BL,S} \) using

\[
\phi_{BL} = C \times \text{USX} \cdot D_{BL,S} / [(\mu_{en}/\rho)_{BL} \times E]
\]

where \( C \) is the conversion coefficient of \( 6.24 \times 10^{15} \text{ eV g}^{-1} \text{Gy}^{-1} \), \( \text{USX} \cdot D_{BL,S} \) is the surface dose rate in Gy \( \text{s}^{-1} \), \( (\mu_{en}/\rho)_{BL} \) is the mass energy absorption coefficient in \( \text{cm}^2 \text{g}^{-1} \), \( E \) is the photon energy in eV (1.5 keV for Al Kα X-rays) (Fulford et al 1999). Since 7% of the photon intensity was attenuated by the 0.75 μm surface layer, the photon fluence rate at the incident surface of HD-810 film (\( \phi_0 \)) was about 1.1\( \phi_{BL} \). Therefore for the USX system used in this study, the photon fluence rate at the center surface of the target (\( \phi_0 \) in photons \( \text{s}^{-1} \text{cm}^{-2} \)) can be estimated for each emission current (I in mA) using

\[
\phi_0 = 1.1 \times 6.24 \times 10^{15} \times 1.29 \times 1.42 / (809 \times 1.5 \times 10^{3}) \times I = 1.0 \times 10^{10} I
\]
One application of this study is to provide the value for the absorbed dose for dry biological samples irradiated under ultra-high vacuum (Boudaïffa et al 2000a, 2000b). Such studies serve to determine the direct effects of ionizing radiation including the fate of secondary electrons (Sanche 2002, Pan et al 2003). We are particularly interested in radiation damage to DNA irradiated in vacuum by Al_kα X-rays. Based on equations 5-7, the mean absorbed dose of USX to DNA with a thickness \( d \) and density \( \rho \) at emission current \( I \) (in mA) can be derived using

\[
\text{USX}_m = \frac{\phi_0 \times (\mu_{en}/\rho)_{DNA} \times E/C \times \{1-\exp(-\mu_{en}/\rho)_{DNA} \times \rho \times d)\} \times ((\mu_{en}/\rho)_{DNA} \times \rho \times d)}{0.0024 \times (1-\exp(-\mu_{en}/\rho)_{DNA} \times \rho \times d)) / (\rho \times d)}
\]  

(8)

For Al_kα X-rays, the calculated mass energy absorption coefficients for the free acid and the sodium salt of dry pGEM-3zf(-) plasmid DNA based on the chemical composition, including the GC content, are \( 9.94 \times 10^2 \) and \( 1.15 \times 10^3 \, \text{cm}^2\,\text{g}^{-1} \), respectively (Hubbell and Seltzer 1995).

4. Concerning the generation and fate of secondary electrons

The active layer of a HD-810 film is sandwiched between gelatin and polyester layers. Based on the mass attenuation coefficients (Hubbell and Seltzer 1995), 6.5 \( \mu \text{m} \) of the active layer was estimated to absorb 41% of the passing Al_kα X-rays. Secondary electrons would be produced in all the three layers by the X-rays and exchanged between layers. These secondary electrons may have an effect on the absorbed doses measured by HD-810 films, but the possible effect was neglected in this study. Therefore, when using HD-810 films to estimate the USX absorbed dose, it is best to place samples on the same polyester film rather than directly on a stainless steel support. GAF films without the
gelatin surface layer and with a thick active layer (thick enough to absorb 100% of USX) would be ideal for USX dosimetry.

Conclusion

In this study, color scanners were found to provide excellent measurement of the optical density of GAF films. The “true color” output was a better choice than “grayscale”. Channel choice and color adjustments allow one to maximize either the sensitivity or the measurable dose range. Readout of HD-810 films from the R channel with the color adjustment set at $x = -100$, $y = 0$ resulted in a linear response to the absorbed doses up to 100 Gy and a sensitivity slightly higher than that of the Nuclear Associate Radiochromic Densitometer Model 37-443. Readout of HD-810 films from the B channel with the color adjustment set at $x = 0$, $y = 100$ extended the measurable dose limit up to 5.5 kGy.

This study provides the first evidence that GAF films are promising secondary dosimeters for measuring the absorbed dose of ultrasoft X-rays. The response of GAF films to the absorbed dose in water was affected by photon energies and determined by the ratio of mass energy absorption coefficients between the active layer of GAF films and water. In the present study, the determination of the absorbed doses of USX was based on a calibration made with $\gamma$-rays (Fricke dosimetry). Further studies on comparing the response of the HD-810 film to USX directly with a primary dosimetry method will be necessary for accurate measurement of the absolute dose for USX.
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Prestwich W V, Murphy R J 2000 Calculated dose response of Gafchromic MD55 film for $^{103}$Pd and $^{125}$I relative to $^{60}$Co *Radiation Measurements* **32** 173-179

II. RESULTS

Verification of brachytherapy dosimetry with radiochromic film Medical Dosimetry 24
197-203

<table>
<thead>
<tr>
<th>Layer</th>
<th>Condition</th>
<th>Density ((\rho) in g cm(^{-3}))</th>
<th>fraction by weight ((f))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H</td>
</tr>
<tr>
<td>Gelatin Surface (0.75 (\mu m))</td>
<td>in air</td>
<td>1.08</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td>in vacuum</td>
<td>1.0</td>
<td>0.091</td>
</tr>
<tr>
<td>Active (6.5 (\mu m))</td>
<td>in air</td>
<td>1.2</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>in vacuum</td>
<td>1.0</td>
<td>0.078</td>
</tr>
<tr>
<td>Polyester Base (99 (\mu m))</td>
<td>in air/vacuum</td>
<td>1.35</td>
<td>0.059</td>
</tr>
</tbody>
</table>

\(^a\) Data was provided by the ISP.
**Table 2. The effect of color adjustment and channel choice on the responses of HD-810 films to the absorbed doses of γ-rays to water**

<table>
<thead>
<tr>
<th>Channel</th>
<th>RGB</th>
<th>Red</th>
<th>Green</th>
<th>Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best setting for color adjustment&lt;sup&gt;a&lt;/sup&gt;</td>
<td>x</td>
<td>0</td>
<td>-100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>-100</td>
<td>0</td>
<td>-100</td>
</tr>
<tr>
<td>Increase of sensitivity&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>50</td>
<td>275</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>Standard deviation (SD) of the pixels in a scanned image</td>
<td>1.8</td>
<td>2.3</td>
<td>1.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Standard deviation of ABS resulting from SD(pixels)</td>
<td>0.013</td>
<td>0.03</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Linear regression</td>
<td>Measurable dose ranges&lt;sup&gt;c&lt;/sup&gt; (Gy)</td>
<td>10 - 120</td>
<td>6 - 100</td>
<td>32 - 250</td>
</tr>
<tr>
<td>ABS = A × Dose</td>
<td>A (kGy&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>2.71 ±0.06</td>
<td>10.0 ± 0.4</td>
<td>2.47 ± 0.03</td>
</tr>
<tr>
<td>Regression fit to Equation 2</td>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.990</td>
<td>0.991</td>
<td>0.996</td>
</tr>
<tr>
<td>ABS = B × [1 - exp (-A × Dose)]</td>
<td>Measurable dose ranges (kGy)</td>
<td>0.01 - 1.0</td>
<td>0.006 - 0.1</td>
<td>0.032 - 0.9</td>
</tr>
<tr>
<td></td>
<td>A (kGy&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>5.3 ± 0.3</td>
<td>NA</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.67 ± 0.01</td>
<td>NA</td>
<td>1.71 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.999</td>
<td>NA</td>
<td>0.999</td>
</tr>
</tbody>
</table>

<sup>a</sup>The best setting for color adjustment was defined as that which resulted in the highest sensitivity at a fixed readout channel.

<sup>b</sup>The increase in sensitivity was defined as the percentage increase of ABS at 0.1 kGy at the optimal setting for color adjustment compared to that with the color adjustment turned off.

<sup>c</sup>The measurable dose range was defined as the doses which produce an ABS ranging from twice the standard deviation (SD) of the ABS resulting from the SD of the pixel determination up to saturation levels.
Table 3 Mass Energy Absorption Coefficients of Water, Three Layers of HD-810 Films ($\mu_{en}/\rho$ in cm$^2$ g$^{-1}$)$^a$ and Attenuation of Photon Intensity ($I/I_0$)$^b$ by Each Component at Three Levels of Photon Energy

<table>
<thead>
<tr>
<th>Component</th>
<th>Condition</th>
<th>Thickness (d in μm)</th>
<th>Photon Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.25 MeV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\mu_{en}/\rho$</td>
</tr>
<tr>
<td>Water</td>
<td>in air</td>
<td>6.5</td>
<td>2.96E-2</td>
</tr>
<tr>
<td>Surface Layer</td>
<td>in air</td>
<td>0.75</td>
<td>2.89E-2</td>
</tr>
<tr>
<td></td>
<td>in vacuum</td>
<td></td>
<td>2.88E-2</td>
</tr>
<tr>
<td>Active Layer</td>
<td>in air</td>
<td>6.5</td>
<td>2.91E-2</td>
</tr>
<tr>
<td></td>
<td>in vacuum</td>
<td></td>
<td>2.91E-2</td>
</tr>
<tr>
<td>Base Layer</td>
<td>in air or vacuum</td>
<td>99</td>
<td>1.00E-2</td>
</tr>
</tbody>
</table>

$^a$ The mass energy absorption coefficient is calculated based on the fraction by weight ($f$ in Table 1) and the coefficient for each component element using $\mu_{en}/\rho = \sum f_i (\mu_{en}/\rho)_i$ (Hubbell and Seltzer 1995).

$^b$I/I$_0$ = exp[-10$^{-4}$.($\mu/\rho$)·$d$], $\rho$ is density in g cm$^{-3}$, $\mu/\rho$ is the mass attenuation coefficient in cm$^2$ g$^{-1}$ and calculated based on $\mu/\rho = \sum f_i (\mu/\rho)_i$ (Hubbell and Seltzer 1995).
Figure 1 Plot of ABS of HD-810 films exposed to γ-rays (○) and soft X-rays (effective energy of 14.8 keV, □) vs the absorbed doses in water. ABS was read from the Red channel with color adjustment set at x = -100, y = 0. The curves were the best fits of the experimental data to equation 1, resulting in $A = (1.00 \pm 0.04) \times 10^2$ Gy$^{-1}$ and $R^2 = 0.991$ for γ-rays (dotted line), $A = (5.01 \pm 0.06) \times 10^3$ Gy$^{-1}$ and $R^2 = 0.9998$ for soft X-rays (solid line), respectively. The error bars show the standard deviation of the ABS resulting from the SD of the pixel determination.
Figure 2 Plot of ABS of HD-810 films exposed to γ-rays vs the absorbed doses in water.

ABS was read from the Blue channel with color adjustment set at x = 0 and y = 100. The curve was the best fit of the experimental data to equation 2, resulting in $A = (0.62 \pm 0.04) \text{ kGy}^{-1}$, $B = 1.24 \pm 0.04$ and $R^2 = 0.998$, respectively. The error bars show the standard deviation of the ABS resulting from the SD of the pixel determination.
Figure 3 Plot of ABS of HD-810 films exposed to ultrasoft X-rays (photon energy of 1.5 keV) vs. the irradiation time as a function of emission currents (○ 18.1, □ 7.2, ◆ 3.8, Δ 1.1 mA, respectively). ABS was read from the Blue channel with color adjustment set at x = 0 and y = 100. The curves were drawn based on equation 2 with $A = 0.62 \text{ kGy}^{-1}$, $B=1.24$ and $Dose = U^{\text{ABS}} D_{\text{BL-m}} \times \text{Time}$. The error bars show the standard deviation of the ABS resulting from the SD of the pixel determination.
**Figure 4** Plot of the mean absorbed dose to the active layer of HD-810 films exposed to Al_{10.6} ultrasoft X-rays vs. the irradiation time as a function of the emission currents (○ 18.1, □ 7.2, ◇ 3.8, △ 1.1 mA, respectively). The error bars showed the standard deviation of the ABS resulting from the SD of the pixel determination. Inserted figure (Fig.4a) shows the plot of the mean absorbed dose rate vs. emission currents. The straight line vertex at the origin is the linear least square fit of experimental data with a slope of (1.42 ± 0.02) Gy s⁻¹ mA⁻¹ and R² = 0.9998.
II. RESULTS

DNA INTER-DUPLEX CROSSLINKS INDUCED BY AlKα X-RAYS UNDER VACUUM

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Proposed running head: DNA INTER-DUPLEX CROSSLINKS

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Dry pGEM\textsuperscript{®}-3Zf(-) plasmid DNA was exposed to Al\textsubscript{Kα} X-rays (1.5 keV) for various times in an ultra high vacuum chamber with mean absorbed dose rates ranging from 1.8 to 41.7 Gy s\textsuperscript{-1}. The different forms of plasmid DNA were separated by neutral agarose gel electrophoresis and quantified by staining and laser scanning. In addition to the bands for supercoiled, nicked circular, linear and concatameric forms of plasmid DNA, two additional bands were observed in X-irradiated samples, migrating at rates similar to those for 8 kb and >10kb linear double stranded DNA, respectively. The digestion of irradiated DNA with the restriction enzymes Eco R I and Pvu I suggests that the two slowly migrating bands were interduplex crosslinked DNA. Alkaline agarose gel electrophoresis of irradiated DNA digested with Eco R I confirmed that the interduplex crosslink was covalent. The exposure curves for the formation of nicked circular, linear, interduplex crosslinked DNA, as well as for the loss of supercoiled and concatameric DNA were determined. Their formation and loss were independent of absorbed dose rates over a 20 fold range. The G values for DNA single strand breaks, double strand breaks and crosslinks were derived to be: 62 ± 6, 5.6 ± 0.6 and 16 ± 4 nmol J\textsuperscript{-1}, respectively. The formation of DNA inter-duplex crosslinks appears to be due to a single event. The mechanism responsible for the formation of DNA interduplex crosslinks is discussed with emphasis on its in vivo implications.
INTRODUCTION

DNA is thought to be the primary target of radiation-induced cell lethality, mutation and carcinogenesis. Radiation induced DNA damage includes DNA strand breaks, base lesions and crosslink formation. DNA single strand breaks (SSB), double strand breaks (DSB) and base lesions have been extensively studied (1-6). DNA-protein (7-10), intrastrand (11-15) and interstrand (9, 16-18) DNA crosslinks (CLs) have also attracted considerable interest. However, interduplex DNA CLs (19) have received little attention. During the last decade there were no reports concerning the induction of DNA duplex-to-duplex CLs by ionizing radiation and only one report for non-ionizing radiation. Powsipisilova and Kypr (19) found that DNA duplex-to-duplex CLs were induced in aqueous ethanol solution by UV light; however, no quantum yield for the formation of CLs was reported. Several decades ago Hagen et al (20) and Lücke-Huhle et al. (21) showed evidence that CLs were an important process when calf thymus DNA was irradiated by X or γ-rays in the solid state. The G values for CLs were derived from the exposure curves. However, high absorbed doses (3-30 kGy by Hagen et al. and 10-50 kGy by Lücke-Huhle et al.) were used in their studies and resulted in the formation of insoluble heavily interlaced DNA. On the other hand, the large molecular weight distribution of calf thymus DNA also causes larger errors in quantification. To our knowledge, these are the only publications on DNA duplex to duplex CLs induced by the direct effects of ionizing radiation. The biological importance as well as accurate G values and mechanisms of radiation-induced interduplex CLs in DNA remain unclear. Although the reports of both Hagen et al and Lücke-Huhle were cited in von Sonntag’s textbook (22), DNA interduplex CLs were either not observed or not looked for in most studies on DNA damage by the direct effects of ionizing radiation (1-6). During DNA replication, recombination and mitosis, the potential exists for interduplex CL induction by ionizing
radiation since these processes involve close interaction between duplexes. The formation of DNA DSB in cells by ionizing radiation may have confounded the detection of interduplex CLs. In any case, given the potential cytotoxicity of these lesions, it is important to develop sensitive assays to verify their formation in cells.

In this work, dry pGEM®-3Zf(-) plasmid DNA (3197 bps) was exposed to Alkα X-rays (1.5 keV) for different periods of time in an ultra high vacuum (UHV) chamber. Different forms of plasmid DNA were separated and quantified with neutral agarose gel electrophoresis, staining and laser scanning. Restriction enzyme digestion and alkaline agarose gel electrophoresis were used to verify the covalent nature of the crosslinks between DNA duplexes. Dose rate effects on the formation of DNA SSB, DSB and CLs were examined. The G values for DNA SSB, DSB and CLs were derived from the respective exposure curves. A one hit mechanism is proposed to explain the formation of DNA interduplex crosslinks.

MATERIALS AND METHODS

DNA preparation and manipulation

pGEM®-3Zf(-) plasmid DNA (3197 bps, Promega) was extracted from E coli DH5α, purified using QIAfilter Plasmid Giga Kit (Qiagen). 91% of the purified plasmid DNA was supercoiled (S) and the rest was either concatemeric (CM, 4%) or relaxed circular (C, 5%).

The DNA pellet was redissolved in distilled and de-ionized water (dd H₂O). 130 μg DNA in 6 μL of dd H₂O was deposited on chemically clean tantalum (Ta) sheets, frozen at −130 °C for 5 minutes, and then lyophilized with a hydrocarbon-free sorption pump under a pressure of 3 mtorrs for one hour. Lyophilized DNA formed a 10 μm thick film with a diameter of 3.0
± 0.2 mm and was transferred to a load lock chamber (5 \times 10^{-6} \text{ torr}) for another hour. The DNA target was delivered directly from the load lock chamber to the UHV chamber (3 \times 10^{-9} \text{ torr}) for X-ray irradiation (0.5-15 minutes). The DNA sample was transferred back to the load lock chamber immediately after irradiation.

Once removed from the load lock chamber, the DNA was immediately dissolved in 10 mM Tris-Cl, pH 7.5. The recovery rate was over 98 %. This manipulation of DNA without X-ray irradiation resulted in the formation of 5 ± 1 % of C and traces of bands which co-migrated with interduplex crosslinked DNA, but which probably resulted from the presence of SSB in CM. Agarose gel electrophoresis analysis showed that the non-irradiated control DNA consists of a concatamer of two relaxed circular DNA (CM-2SSB, 0.8 ± 0.6 %), a concatamer of one supercoiled and one relaxed circular DNA (CM-SSB, 0.7 ± 0.5 %), C (10 ± 1 %), CM (4.3 ± 0.8 %) and S (85 ± 2 %).

**X-ray irradiation**

\( \text{Al}_{k\alpha} \) X-rays (1.5 keV) were produced using a PHI Model 04-548 Dual Anode X-ray Source (Perkin Elmer) with an aluminum target. The anode voltage was kept constant at 4 kV. The incident fluence rate of photons (\( \phi_0 \)) was adjustable by the anode emission current ranging from 1.1 mA to 18.1 mA and measured with GAFCHROMIC® HD-810 radiochromatic dosimetry films (23). The incident angle of the X-ray beam with respect to the plane of target surface was 19°. The mean absorbed dose rate of \( \text{Al}_{k\alpha} \) X-rays in DNA was calculated with equation 1:

\[
\dot{D}_m = A \phi_0 (\mu_{\text{ew}}/\rho) E \{1-\exp[-(\mu/\rho)pd]\}/[(\mu/\rho)pd]
\]

(1)
where $A$ is the conversion coefficient of $6.24 \times 10^{15} \text{ eV}^{-1} \text{ g Gy}$, $\phi_0$ is incident fluence rate of photons in photons $\text{s}^{-1} \text{ cm}^{-2}$ ($0.17-3.9 \times 10^{11}$ photons $\text{s}^{-1} \text{ cm}^{-2}$ in this study), $E$ is the photon energy of 1500 eV, $\rho$ is DNA density of 1.46 g cm$^{-3}$, $d$ is the thickness of DNA that X-rays pass in cm ($10 \mu\text{m} / \sin (19^\circ) = 31 \mu\text{m}$ in this study), $\mu_{en}/\rho$ and $\mu/\rho$ are the mass energy absorption coefficient and mass attenuation coefficient of DNA in cm$^2$ g$^{-1}$, respectively. The pGEM$^\text{®}$-3Zf(−) plasmid DNA used in this study has a sodium counterion but without extra salt added and is assumed to have 2.5 bound waters per nucleotide under UHV. Thus, for 1.5 keV X-rays, $\mu_{en}/\rho$ and $\mu/\rho$ are calculated to be 1174 and 1179 cm$^2$ g$^{-1}$, respectively (24). Details of the apparatus have been described elsewhere (26).

Agarose gel electrophoresis

The different forms of double stranded plasmid DNA were separated by 1 % neutral agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 6.7 V cm$^{-1}$ for 7 minutes and 5.0 V cm$^{-1}$ for 75 minutes. Both the gel and the DNA were prestained with SYBR$^\text{®}$ Green I (Molecular Probes). SYBR$^\text{®}$ Green I, supplied as 10000X concentrate in DMSO, was diluted to 1X and 20X with TAE buffer for gel and DNA samples, respectively (25). The single stranded linearized plasmid DNA (cut with the restriction enzyme Eco R1) was separated by 1 % alkaline agarose gel electrophoresis in 50 mM NaOH and 10 mM EDTA at 2.0 V cm$^{-1}$ for 7 hours. After electrophoresis, the gel was soaked in neutralizing solution (1M Tris-Cl, pH 7.6 $+ 1.5$M NaCl) for 45 minutes at room temperature. The neutralized gel was stained with 1X SYBR$^\text{®}$ Gold Nucleic Acid Gel Stain (Molecular Probes) in TAE buffer for 40 minutes at room temperature. For both neutral and alkaline gels, 100 ng of DNA was loaded for each lane. Both types of gel were scanned with a STORM860 in blue fluorescence mode (Molecular Dynamics) with an excitation
wavelength of 450 nm. The relative amount of DNA in each form was quantified with ImageQuant (Molecular Dynamics). A factor of 1.7 was used to correct for the weaker binding of SYBR® Green I to the S than the C and linear (L) forms of plasmid (25).

Calculation of $G$ values

The absolute amount of plasmid DNA in all forms was determined with UV absorption at 260 nm, pH 7.0, taking $\varepsilon = 7120$ mol$^{-1}$ dm$^3$ cm$^{-1}$. The relative amount of the different forms of plasmid DNA was obtained from gel electrophoresis. Scheme 1 shows the reaction pathways for the formation of different DNA products.

Scheme 1 Paths for the formation of circular (C), linear (L) and crosslinked (S-S, S-C, C-C) DNA

The concatamers CM, CM-SSB and CM-2SSB may co-migrate with crosslinked DNA S-S, S-C and C-C, respectively. However, as CM only represents $4.3 \pm 0.8$ \% in control DNA, the contribution of CM-SSB and CM-2SSB to the band of S-C and C-C was ignored in this study. Therefore the $G$ values (in nmol J$^{-1}$) for the formation of SSB, DSB, S-C and C-C as well as the loss of S and CM were derived from the initial linear slopes ($A$ in \% kGy$^{-1}$) of the respective dose response curves given by:

$$G = 10^9 \frac{A}{f} / MW$$

(2)
where \( f \) is the percentage of S (85 %) in control DNA for the formation of SSB, DSB, S-S, S-C and C-C as well as the loss of S and the percentage of CM (4.3 %) in control DNA for the loss of CM; MW is the molecular weight of pGEM\(^\text{\scriptsize R}^\text{-}3Zf(-)\) plasmid DNA sodium salt (2.1 \( \times \) 10\(^6\) daltons) for SSB and DSB and the loss of S, of plasmid DNA dimer (4.2 \( \times \) 10\(^6\) daltons) for C-C and S-C and the loss of CM, respectively.

The \( G \) value for the formation of S-S was estimated by comparing \( G(-S) \) and \( G(-CM) \).

Since S-S may co-migrate with CM,

\[
A(CM^{\text{App}}) = A(CM) - A(S-S)
\]

(3)

According to equation (2),

\[
4.3 \ G(CM^{\text{App}}) = 4.3 \ G(CM) - 85 \ G(S-S)
\]

(4)

Theoretically \( G(-CM) = G(-S) \), thus

\[
G(S-S) = 4.3 / 85 \times [G(S)-G(CM^{\text{App}})]
\]

(5)

**DNA digestion with restriction endonucleases**

1 \( \mu \)g of plasmid DNA was incubated either with 8 Units of Eco R1 restriction endonuclease (New England Biolabs) in NEBuffer 1 (10 mM Tris -HCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol, pH 7.0) or with 10 Units of Pvu I restriction endonuclease (New England Biolabs) in NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol, pH 7.9) supplement with 100 \( \mu \)g/ml BSA at 37\(^\circ\)C for 2 hours.

**RESULTS AND DISCUSSION**

1. *Identification of inter-duplex covalently crosslinked DNA*
II. RESULTS

Figure 1 shows a scanned image of a 1% neutral agarose gel, with a 1 kb DNA ladder (Lane 1), non-irradiated plasmid DNA (Lane 2), X-ray irradiated DNA (mean absorbed dose: 3.3 kGy, Lane 3), Eco R1 digested non-irradiated and irradiated DNA (Lane 4 and 5), Pvu I digested control and irradiated DNA (Lane 6 and 7). Lane 2 shows that the non-irradiated DNA consists of C (5%), CM (4%) and S (91%). As shown in lane 3, exposure to X-rays results in the loss of S (31%) and CM (2%), the formation of C (22%), L (2%), and two unknown forms of DNA (X1, 5%; X2, 4%). X1 and X2 migrated at rates similar to those of 8 kb and >10 kb linear DNA, slower than C and CM. Since pGEM®-3Zf(-) plasmid DNA has 3197 bps, X1 and X2 probably represent dimers of two nicked circular plasmids (C–C) for X1, or one supercoiled and one nicked circular DNA (S–C) for X2. Theoretically, the DNA duplex to duplex CLs should have 3 forms: S–S, S–C and C–C. However only two additional bands were easily observed, probably because S–S and CM are both dimers of two supercoiled plasmids and likely migrate at the same rate, even though the two DNA duplexes are chemically bonded for S–S, but are interlocked for CM. In order to test the above assumption, both control and irradiated DNA were digested with restriction endonucleases Eco R1 and Pvu I, respectively. Eco R1 recognizes the sequence: 5’...G^AATTC...3’ and cuts pGEM®-3Zf(-) only once at nucleotide 5. Pvu I recognizes the sequence: 5’...CGAT^CG...3’ and cuts pGEM®-3Zf(-) twice at nucleotides 1708 and 3058, respectively. As expected and shown in lanes 4 and 5, Eco R1 converts all three topological forms (C, CM and S) in control DNA into linear DNA. In contrast, upon Eco R1 digestion of irradiated DNA, the S–C band disappeared, but the C–C band increased in intensity. This result suggests that C–C and S–C are crosslinked DNA dimers, which can not be converted into L by Eco R1. Lanes 6 and 7 show that Pvu I cuts pGEM®-3Zf(-) plasmid twice and
converts it to two linear fragments of 1350 bps (F1) and 1847 bps (F2), respectively. Pvu I digestion also converted the S-C and C-C to three closely spaced bands, the migration rates of which are similar to those of 4-5 kb linear DNA. Theoretically, Pvu I should cut S-C and C-C into three X-shaped fragments: F1-F1 (2700 bps), F1-F2 (3197 bps), F2-F2 (3694 bps) with yields of 1:2:1, respectively. The inserted chromograph supports this prediction. These results suggest that C-C and S-C are dimeric rather than multimeric DNA.

Figure 2 shows a scanned image of a 1% alkaline agarose gel with Lambda DNA-Hind III digest (Lane 1), 1 kb DNA ladder (Lane 2), non-irradiated plasmid DNA -Eco R1 digest (Lane 3) and X-ray irradiated DNA -Eco R1 digest (mean absorbed dose: 3.6 kGy, Lane 4). Lane 3 shows that the non-irradiated DNA only consists of one linear fragment of about 3.2 kb. As shown in lane 4, exposure to X-rays resulted in the formation of one band which migrated at a rate similar to the 6.6 kb fragment of the Lambda DNA-Hind III digest. This result confirms that X-rays indeed induce the formation of covalently crosslinked dimers. Both bands of lane 4 have long tails, suggesting that the sites for both strand breaks and crosslinks are random.

Hagen et al. (20) observed the formation of highly interlaced DNA (5-15 interlaced DNA) molecules by 10 keV X-ray irradiation of dry calf thymus DNA under vacuum. The molecular weight distribution was determined by analytical ultracentrifugation. Higher doses resulted in a larger fraction of heavily interlaced DNA (>15 DNA molecules). The calf thymus DNA was linear, with an average length >13kb. The longer length and the more flexible linear form of the calf thymus DNA compared to plectonemically supercoiled plasmid DNA may be responsible for the higher degree of interlacing observed in Hagen et al.’s study than in the present study.
DNA duplex-to-duplex CLs were reported to be induced in aqueous ethanol solution by UV light (19). The effects of NaCl and ethanol concentration on the formation of inter-duplex CLs suggested that the crosslinking was specific to the A conformational form of DNA induced by high concentration of ethanol. Crystallographic studies of DNA fragments of the A and B conformations show that the A-DNA minor groove provides sites for intermolecular contacts through hydrophobic and polar interactions (27). Gaillard et al found that DNA molecules bound to polypropylene adopt a conformation with locally unwound regions, which allows interactions and pairing between neighboring duplexes and results in multimeric DNA (28). The DNA used in the present study underwent freeze drying on tantalum under vacuum before exposure to X-rays. It is highly likely that our DNA was transformed from the B to A conformation during the dehydration process and in addition, may have locally unwound regions. This suggests that a conformational transition may be the key for the formation of inter-duplex CLs.

2. Absorbed dose dependence of the formation of DNA SSB, DSB and CLs

The exposure curves of the formation of C (SSB), L (DSB), C-C (CL1) and S-C (CL2) DNA as well as the loss of S and CM were determined for the mean absorbed dose up to 12 kGy and are shown in Fig. 3a and 3b. The formation of C, L, C-C and S-C as well as the loss of S and CM increase linearly as the mean absorbed dose rises up to 0.8 kGy (Fig. 3c and 3d), then become saturated at higher doses. From the slopes of the respective initial linear dose response curves, the G values (in nmol J⁻¹) for the formation of C, L, C-C and S-C as well as the loss of S and CM were derived to be 62 ± 6, 5.6 ± 0.6, 7 ± 1, 6 ± 2, 99 ± 14 and 53 ± 21, respectively. Theoretically the G value for the loss of S and CM should be the
same. The significantly smaller G value for the loss of CM than S can be explained by the co-migration of CM and S-S. Therefore the G value for the formation of S-S was estimated to be $3 \pm 1$ nmol J$^{-1}$ using equation 5. Thus, the G value for all CLs is $16 \pm 4$ nmol J$^{-1}$. For clarity, the G values for the formation of C, L, S-S, S-C and C-C are compiled in Table 1. The ratio of SSB, DSB and total CLs is 1: 0.09: 0.25. This result suggests that crosslink formation is a process at least as important as DSB for X-ray induced DNA damage in the solid state, in agreement with the findings of Hagen and Wellstein (20) and Lücke-Huhle et al (21). The G value for total CLs ($16 \pm 3$ nmol J$^{-1}$) derived in the present study is somewhat smaller than the value obtained by Hagen and Wellstein ($37$ nmol J$^{-1}$ under vacuum)(20), but agrees well with that by Lücke-Huhle et al ($18$ nmol J$^{-1}$ under nitrogen) (21). There are several differences between these two studies and ours. First, Hagen and Wellstein used 10 keV X-rays and Lücke-Huhle et al employed γ-rays, while we used 1.5 keV ultrasoft X-rays. Second, they derived the G values from much higher absorbed doses [3-30 kGy (20) or 10-50 kGy (21)] than used in the present study (< 0.8 kGy). Third, multimeric interlaced DNA was formed in both published studies, while only dimeric DNA was observed in our study.

Since the reports of Lücke-Huhle et al in 1965 and 1970, respectively (20,21), there have been many reports concerning SSB and DSB formation in dry DNA induced by γ-rays (1, 2) and X-rays (3 - 6) of various energies under atmospheric or vacuum conditions, but surprisingly there have been no reports of γ- or X-ray induced interduplex crosslinks in dry DNA. The DNA used in the most studies (1-4) contained salts or buffers such as Tris-HCl, EDTA-Na or NaCl, which can account for more than 30 % of the total sample weight. The salt or buffer may alter the conformation of dry DNA, thus reducing the possibility of interaction and crosslinking between neighboring duplexes. It is also possible that the salt or
buffer block the interaction of the radical formed in one duplex with an adjacent duplex, although we have not examined the effects of salt or buffer in our system. Ito et al noted that the presence of NaCl in dry DNA (the ratio of NaCl vs DNA by weight = 0.8) prior to irradiation was necessary in order to dissolve dry DNA films following irradiation (2). This suggests that very heavy crosslinking might have been formed in dry DNA irradiated in the absence of NaCl, preventing its dissolution, although this was not specifically tested. For the crystalline DNA used by Razskazovskiy et al. (6), inter-duplex interaction is greatly restricted. This or its unusual Z conformation might account for the fact that no interduplex CL induction by X-rays was noted in their study.

The G value (in nmol J\(^{-1}\)) for SSB (62 ± 6) in dry DNA obtained in this study agrees well with literature values: 29-30 for 250 and 380 eV X-rays (5), 28-32 for 2 keV X-rays (4), 63-340 for 10 keV X-rays (20), 70-160 for 70 keV X-rays (6), 56, 72-140 for γ-rays (1, 21) and 167-170 for 388, 435,573 eV X-rays (3), considering the differences in DNA samples (content of salts and buffers), radiation sources and irradiation conditions (under air or vacuum). Similarly, the ratio (=11) of G values for SSB vs DSB obtained in this study also nicely falls within the range of reported values: 5-6 for 250 and 380 eV X-rays (5), 5.7 for 10 keV X-rays (20), 10 or 5.5 for γ-rays (1, 21), 17-19 for 388, 435,573 eV X-rays (3), 21-26 for 2 keV X-rays (4).

3. Dose rate effects on the formation of SSB, DSB and CLs

It is possible that certain products, such as inter-duplex crosslinked DNA, result from the interaction of two reactive radiolysis intermediates. This would give rise to a dose rate effect, as discussed by Briskman et al (29). To investigate whether DNA SSB, DSB and CLs are
induced by one or two X-ray photon hit events, the dose rate effect on their formation and the loss of S and CM was studied. The mean absorbed dose of DNA was kept constant at 3.6 kGy, while the mean absorbed dose rates were varied from 1.8 to 41.7 Gy s\(^{-1}\). As shown in Fig. 4, the formation of DNA SSB, DSB and CLs and the loss of S and CM are independent of the mean absorbed dose rate. This suggests that DNA SSB, DSB and CLs all resulted from single hit rather than two hit events. The linear induction of CLs as a function of the absorbed doses (Fig. 3d) also supports the single hit mechanism.

The study of SSB formation in crystalline DNA induced by X-rays (6) suggests that SSB are generated by direct ionization of DNA which gives rise to a set of sugar radicals. Sanche and his coworkers reported that low energy secondary electrons induce DNA SSB and DSB by dissociative electron attachment, probably involving transient sugar radicals (30-32). The formation of S-C and C-C suggests that transient sugar radicals related with DNA SSB might be involved in the interduplex crosslinking. A sugar radical might add to a double bond of DNA bases at a locally unwound region of a neighboring DNA duplex, resulting in interduplex CLs. The formation of S-S implies that an alternative pathway for DNA interduplex crosslinking might only involve base radicals. Multimeric concatamers were reported to be formed in vitro during recombination (33,34) and replication processes (35) where locally unwound region of DNA duplex exists. It will be important to study whether and how X-rays might induce DNA interduplex crosslinks in vivo during recombination, replication and chromosome condensation, as well as whether and how the crosslinks are repaired. On the other hand, the formation of DNA interduplex CLs induced by X-rays may also serve as a sensitive probe for the presence of locally unwound regions of DNA duplex.
II. RESULTS

In conclusion, we found that Alka X-rays induced DNA SSB, DSB and inter-duplex CLs in dry DNA under UHV. The G value for total DNA inter-duplex CLs is $16 \pm 4 \text{ nmol J}^{-1}$, which is 0.25 and 2.8 fold that of the G values for DNA SSB and DSB, respectively. The inter-duplex crosslinks appear to be formed via a one-hit event. DNA conformations allowing interaction of neighboring DNA duplexes, such as locally unwound DNA during recombination and replication, may be necessary for the induction of DNA inter-duplex crosslinks. The possibility of DNA interduplex crosslink induction by radiation in vivo should be investigated, given the potential toxicity of such lesions.

ACKNOWLEDGMENTS

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REFERENCES


Figure 1 Neutral agarose gel electrophoresis of pGEM®-3Zf(-) plasmid DNA: effects of X-irradiation, digestion with Eco R1 and Pvu I restriction enzymes. For lane 3, the bands from the top to the bottom (migrating slowest to fastest) are crosslink 1 (C-C), crosslink 2 (S-C), circular (C), linear (L), concatameric (CM) and supercoiled (S) DNA. The insert shows the profile of the framed bands.
Figure 2 Alkaline agarose gel. Lanes from the left: 1) Lambda DNA-Hind III digest  2) 1 kb DNA ladder 3) non-irradiated pGEM®-3Zf(-) plasmid DNA - Eco R1 digest 4) X- irradiated 3Zf(-) plasmid DNA- Eco R1 digest.
Figure 3 The dependence of the formation of circular (C), crosslink 1 (C-C), crosslink 2 (S-C) and linear (L) DNA as well as the loss of supercoiled (S) and concatameric (CM) DNA on mean absorbed doses in DNA. Dose range: 0-12 kGy (a and b); 0-0.8 kGy (c and d). The error bars show the standard deviation of 4 experiments.
Figure 4 The effect of mean absorbed dose rate on the formation of circular (△), crosslink 1 (□), crosslink 2 (○), linear (▽) DNA and the loss of supercoiled (●) and concatameric (◇) DNA. The mean X-ray absorbed dose was 3.6 kGy. The error bars show the standard deviation of 4 experiments.
Comparison between X-ray photon and secondary electron damage to DNA in vacuum

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ABSTRACT: Both monolayer and thick (20 μm) films of dry pGEM\textsuperscript{®}-3Zf(-) plasmid DNA deposited on tantalum foil were exposed to Al\textsubscript{κα} X-rays (1.5 keV) for various times in an ultra high vacuum chamber. For monolayer DNA, the damage was induced mainly by low energy secondary electrons (SEs) emitted from the tantalum. For the thick films, DNA damage was induced chiefly by X-ray photons. Different forms of plasmid DNA were separated and quantified by agarose gel electrophoresis. The exposure curves for the formation of nicked circular (single strand break, SSB), linear (double strand break, DSB), interduplex crosslink forms 1 and 2 were obtained for both monolayer and thick films of DNA, respectively. The lower limits of G values for SSB and DSB induced by SEs were derived to be: 86 ± 2 and 8 ± 2 nmol J\textsuperscript{-1}, respectively. These values are 1.5 and 1.6 times larger than those obtained with 1.5 keV photons. The projected X-ray energy dependence of the low energy electron (LEE) enhancement factor for the SSB and DSB in monolayer DNA is also discussed. This new method of investigation of the SE-induced damage to large biomolecules allows direct comparison of the yield of products induced by high energy photons and LEEs under identical experimental conditions.
INTRODUCTION

It is well known that ionizing radiation induces DNA strand breaks (SBs) by direct ionization and excitation of DNA\textsuperscript{1-6} or by indirect chemical reaction of products of water radiolysis, mainly hydroxyl radicals, with DNA\textsuperscript{7}. It is also known that solvated electrons primarily attack DNA bases and do not result in SBs\textsuperscript{8}. Only recently has it been demonstrated that electrons with very low energies (even close to 0 eV) can also induce SBs in dry DNA under vacuum conditions\textsuperscript{9-14}. Ionizing radiation generates very large quantities of secondary electrons (SEs) in cells (~ 40,000 are produced by a 1 MeV electron) with a most probable energy lying below 10 eV\textsuperscript{15,16}. It is therefore important to quantitatively compare the efficiency of DNA damage induced by low energy electrons (LEEs) and ionizing radiation. Although there are numerous G values for DNA SBs induced by various types of ionizing radiation\textsuperscript{1-6,8}, only a few quantum yields for DNA strand breaks induced by LEEs\textsuperscript{10, 11, 14} are available in the literature. Furthermore, the comparison of the values obtained from these different types of experiments is difficult, mainly because the experimental conditions, including the composition and conformation of the DNA, are different. In addition, the dosimetry for LEE beam experiments is not available due to problems related to charging and the energy imparted both to the DNA film and the metal substrate\textsuperscript{9,12}. This paper presents the first direct comparison of DNA damage induced by high energy photons (Al\textsubscript{K}α X-rays of 1.5 keV) and LEEs (average energy of 5.8 eV) under identical experimental conditions. Such a comparison is made technically possible due to the large amount of X-ray induced low energy SEs emitted from metal surfaces. The energy spectra of the emitted electrons typically exhibit a
narrow peak below 10 eV for the low energy part and relatively sharper elastically scattered photoelectron and Auger electron peaks at high energy\textsuperscript{17}.

**MATERIALS AND METHODS**

*DNA preparation and manipulation*

pGEM\textsuperscript{®}-3zf(-) plasmid DNA (3197 bps, Promega) was extracted from E.coli DH\textsubscript{5α}, purified with QIAfilter Plasmid Giga Kit (Qiagen). Agarose gel electrophoresis analysis showed that 95 % of the purified plasmid DNA was in the supercoiled form and the rest was in the concatemeric (4 %) and nicked circular (1 %) forms. The DNA pellet was redissolved in distilled and de-ionized water (dd H\textsubscript{2}O). Under a dry atmosphere, either 43.6 \textmu g of DNA in 2 \textmu L of dd H\textsubscript{2}O or 100 ng of DNA in 20 \textmu L of ddH\textsubscript{2}O was deposited on a chemically clean tantalum (Ta) sheet, frozen at −130 °C for 5 minutes, then lyophilized with a hydrocarbon-free sorption pump under a pressure of 3 mtorrs for one hour. The former lyophilized DNA created a 20 \textmu m thick film with a diameter of 1.3 ± 0.2 mm, while the later formed a monolayer with a diameter of 6.0 ± 0.2 mm. The lyophilized DNA was transferred to a load lock vacuum chamber which reached a pressure of 5 \times 10\textsuperscript{-6} torr after an hour. This chamber is evacuated by an oil-free turbo-molecular pump and connected to an ultra-high vacuum (UHV) chamber via a gate valve. It is equipped with a sample holder connected to a mechanical feedthrough, which allows transfer of the sample, via the gate valve, to another target holder located in the UHV chamber. With this system, it is possible to introduce the DNA sample into the UHV chamber for X-ray exposure without breaking the \sim 3\times10\textsuperscript{-9} torr vacuum. Details of the apparatus have been described elsewhere\textsuperscript{18}.
After 0 -15 minutes of X-ray irradiation, the sample was transferred back to the load lock chamber. Once removed from the latter, the DNA was immediately dissolved in 10 mM Tris-Cl, pH 7.5. The recovery rate was over 98 %. These same manipulations of DNA without X-ray irradiation resulted in the formation of 5 ± 1 % of nicked circular DNA and 0.5 ± 1 % of DNA which co-migrated with dimeric DNA, but no linear DNA.

**X-ray irradiation and dosimetry**

$\text{Al}_{k\alpha}$ X-rays (1.5 keV) were produced using a PHI Model 04-548 Dual Anode X-ray Source (Perkin Elmer) with an aluminum target. For all experiments, the anode voltage and emission current were kept constant at 4 kV and 1.1 mA, respectively. The incidence of the X-rays was normal to the target surface. The incident fluence rate of photons ($\phi_0$) was determined with GAFCHROMIC® HD-810 radiochromatic dosimetry films to be $1.14 \times 10^{10}$ photons s$^{-1}$ cm$^{-2}$. The mean absorbed dose rate in DNA of $\text{Al}_{k\alpha}$ X-rays was calculated with equation 1:

$$\dot{D}_m = C \phi_0 (\mu_{em}/\rho) E \{1-\exp[-(\mu/\rho)d]\}/[(\mu/\rho)d]$$

(1)

where $C$ is a conversion coefficient of $1.6 \times 10^{-16}$ g Gy eV$^{-1}$. $\phi_0$ is the incident fluence rate of photons in photons s$^{-1}$ cm$^{-2}$, $E$ the photon energy in eV, $\rho$ the DNA density in g cm$^{-3}$ (1.46 g cm$^{-3}$ in this study) and $d$ the thickness of DNA in cm; $\mu_{em}/\rho$ and $\mu/\rho$ are the mass energy absorption coefficient and the mass attenuation coefficient of DNA in cm$^2$ g$^{-1}$, respectively. The sodium salt of pGEM®-3Zf(-) plasmid DNA used in this study was assumed to have 2.5 bound water per nucleotide. Thus, for 1.5 keV X-rays, $\mu_{em}/\rho$ and $\mu/\rho$ of DNA are calculated to be 1174 and 1179 cm$^2$ g$^{-1}$, respectively.
**II. RESULTS**

*X-ray induced SE emission from the tantalum surface*

The number of SEs emitted from the tantalum surface induced by Al$_{K\alpha}$ X-rays was measured as sample current (I, in nA) using a Keithley 610C solid state electrometer connected between tantalum target (1.4 × 1.4 cm$^2$) and ground.

The relative energy distribution of fast SEs ($E_k = 10 – 1486$ eV), was measured with an analytical X-ray photoelectron spectrometer (XPS, Perkin Elmer). For slow SEs ($E_k \leq 10$ eV), the energy distribution was calculated using equation 2$^{21}$:

$$\eta(E_k) = \eta_e E_k^4 / (E_k + W)^4$$  \hspace{1cm} (2)

where $\eta(E_k)$ and $\eta_e$ are the yields of SEs having kinetic energy of $E_k$ and 0-10 eV, respectively; $W$ is the work function of tantalum, *i.e.* 4.12 eV$^{22}$.

*Agarose gel electrophoresis*

Various forms of double stranded plasmid DNA were separated by 1 % agarose gel electrophoresis run in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 6.7 V cm$^{-1}$ for 7 minutes and 5.0 V cm$^{-1}$ for 75 minutes$^{23}$. Both gel and DNA were prestained with SYBR$^{\text{®}}$ Green I (Molecular Probes) (1X for gel and 20X for DNA, respectively). 100 ng of DNA was loaded into each well. After electrophoresis, the gel was scanned with the STORM860 using Blue Fluorescence mode (Molecular Dynamics) at an excitation wavelength of 450 nm. The relative amount of DNA in each form was quantified with ImageQuant (Molecular Dynamics). A factor of 1.7 obtained from previous studies$^{23}$ was used to correct for the weaker binding of SYBR$^{\text{®}}$ Green I to the supercoiled form of DNA compared to nicked circular and linear configurations.
Calculation of G values

The absolute amount of plasmid DNA in all forms was determined by measuring its UV absorption at 260 nm, taking $\varepsilon = 7120\ \text{mol}^{-1}\ \text{dm}^3\ \text{cm}^{-1}$ at pH 7.0. The relative amounts of the different forms of plasmid DNA was obtained from agarose gel electrophoresis.

The G values ($G^p$ in mol J$^{-1}$) for the formation of circular (C, SSB), linear (L, DSB), crosslink 1 (S-C, CL1) and crosslink 2 (C-C, CL2) DNA as well as the loss of supercoiled (S) and concatemeric (CM) DNA$^{25}$ induced by X-rays were derived from the initial linear slopes (A in % kGy$^{-1}$) of the respective dose response curves given by:

$$G^p = \frac{A}{(f \times MW)}$$  \hspace{1cm} (3)

where MW is the molecular weight of pGEM$^{\circledR}$-3Zf(-) plasmid DNA sodium salt (2115979 Da) for the formation of SSB and DSB and the loss of S. For the formation of CLs and the loss of CM, MW is the molecular weight of plasmid DNA dimer (4231958 Da). f is the percentage of S in control DNA for the formation of SSB, DSB, S-C and C-C as well as the loss of S, and the percentage of CM in control DNA for the loss of CM.

RESULTS AND DISCUSSION

SE emission from tantalum: the yield and distribution

Figure 1 Energy spectra of Al$\alpha$ X-ray induced secondary electron emission from tantalum
The current of SEs emitted from the 1.4 \times 1.4 \text{ cm}^2 \text{ tantalum target} was measured to be 0.14 \pm 0.02 \text{ nA} \text{ at an X-ray anode emission current of 1.1 mA.} \text{ Thus the electron flux was } (4.5 \pm 0.3) \times 10^8 \text{ electrons s}^{-1} \text{ cm}^2. \text{ This gave } 0.039 \pm 0.003 \text{ electrons per photon as the total energy-integrated electron yield } (\eta_0), \text{ i.e., the number of emitted SEs of the different kinetic energies per incident photon. This value is in excellent agreement with 0.037 electrons per photon, which was calculated based on the work of Henke et al}^{17}. \text{ Figure 1 shows the energy spectrum of Al}_{k\alpha} \text{ X-ray induced SE emission from tantalum, which has a peak at 1.4 eV and an average energy of 5.8 eV. This is obtained from XPS measurement for } E_k \geq 10 \text{ eV and calculations for } E_k \leq 10 \text{ eV based on equation 2. } \eta(\leq10\text{eV}) \text{ and } \eta(\geq10\text{ eV}) \text{ are derived to be 0.0364 and 0.0028 electrons/photon, respectively. The average energy for SEs with energies less than 10 eV is } 3.0 \text{ eV.} \text{ Time of exposure by X-rays (minutes):} \\
\begin{array}{cccccc}
0 & 1 & 2 & 3 & 4 & 5 & 0 (aq) \\
\end{array} \\
\begin{array}{cccccccc}
\hspace{1cm} & \hspace{1cm} & \hspace{1cm} & \hspace{1cm} & \hspace{1cm} & \hspace{1cm} & \hspace{1cm} & \hspace{1cm} \\
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\end{array} \\
\text{Figure 2} \text{ Agarose gel electrophoresis of DNA following irradiation by Al}_{k\alpha} \text{ X-rays as a monolayer on tantalum foil. The bands starting from the top are: crosslink 1, crosslink 2, circular, linear, concatemeric and supercoiled DNA, respectively. The last lane shows DNA which did not go through any manipulations.}
Figure 2 shows a scanned image of a 1 % agarose gel following electrophoresis of 100 ng of monolayer DNA exposed to $\text{Al}_{\text{K}\alpha}$ X-rays in UHV for 0, 1, 2, 3, 4 and 5 minutes (lane 0 to 6, respectively). For comparison, lane 0(aq) shows 100 ng of DNA which was kept in dd $\text{H}_2\text{O}$ and did not go through any sample manipulations. Six forms of plasmid DNA were separated by the gel. Starting from the top, the bands represent interduplex crosslink form 1 (C-C), form 2 (C-S), nicked circular (C), linear (L), concatemeric (CM), and supercoiled (S) DNA. Verification that the crosslinked plasmids result from covalent interduplex crosslinks has been presented elsewhere.$^{23}$ Manipulation of DNA resulted in the formation of $11 \pm 2 \% \ C$, $0.6 \pm 0.2 \% \ C$-C and $0.4 \pm 0.2 \% \ C$-S as well as the loss of $13 \pm 3 \% \ S$ and $0.3 \pm 0.2 \% \ CM$. As the X-irradiation time increased, the loss of $S$ and $CM$ as well as the formation of $C$-C, $C$-S, C and L increased. The loss of $S$ resulted in the formation of $C$ via SSB, L via DSB, C-C, C-S and S-S via DNA CL. S-S was not observed because it co-migrates with CM. The loss of CM via SSB would result in products co-migrating with C-S or C-C. However, this contribution was ignored in the analysis, because CM only accounts for less than 5 % of all DNA forms.

Similar gel images were obtained for thick films of DNA samples. In this case, manipulation of thick films of DNA resulted in the formation of $8 \pm 1 \% \ C$, $0.4 \pm 0.3 \% \ C$-C and $0.7 \pm 0.5 \% \ C$-S as well as the loss of $10 \pm 3 \% \ S$ and $0.5 \pm 0.5 \% \ CM$. Control experiments on monolayer and thick films of DNA, in the absence of radiation, suggests that the interaction between DNA and tantalum surface results in SSB but not in DSB and CL.

*Dependence of DNA SSB, DSB and CL formation on absorbed dose*
II. RESULTS

Figure 3 shows the linear dependence of the loss of S and CM as well as the formation of C, L, C-C and S-C in thick film and monolayer DNA on the mean X-ray-absorbed dose in DNA, calculated from Eq. 1. The G values were derived from the slope of a linear least squares fit of respective exposure curves and compiled in Table 1.
Figure 3 X-ray exposure curves for the loss of supercoiled (a) and concatemeric (b) DNA as well as the formation of circular (c), linear (d), crosslink form 1 (e) and 2 (f) when DNA was irradiated in a thick film (Δ) and a monolayer (●).
**Table 1** G value (in nmol J\(^{-1}\)) for the formation and the loss of different forms of DNA induced by X-ray photons and secondary electrons

<table>
<thead>
<tr>
<th>DNA</th>
<th>Thick film</th>
<th>Monolayer</th>
<th>Monolayer</th>
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<tr>
<td>Radiation</td>
<td></td>
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<tr>
<td>Photons (G(^P))</td>
<td>Photonsa</td>
<td>Apparent</td>
<td>Secondary electrons</td>
</tr>
<tr>
<td>Circular (SSB)</td>
<td>57 ± 1</td>
<td>90 ± 1</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>Linear (DSB)</td>
<td>5.0 ± 0.4</td>
<td>8 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>C-C (CL1)</td>
<td>4.7 ± 0.2</td>
<td>2.3 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>S-C (CL2)</td>
<td>5.2 ± 0.2</td>
<td>2 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>Supercoiled</td>
<td>- 80 ± 1</td>
<td>-101 ± 15</td>
<td>-50 ± 16</td>
</tr>
<tr>
<td>Concatemeric</td>
<td>-19.4 ± 5</td>
<td>-57 ± 21</td>
<td>0</td>
</tr>
</tbody>
</table>

a Assuming all the energy of the absorbed photons was imparted to DNA and no damage was induced by SEs emitted from tantalum;

b Assuming all the energy of the photons interacting with DNA (0.05% of incident X-rays) was absorbed by DNA and all the energy of SEs emitted from tantalum surface was absorbed by DNA, using \( L_G^{SE} = (G^{AP} - G^P) \times 3.21 / 1.17 \).

c Assuming all the energy of SEs emitted from tantalum surface was absorbed by DNA and no damage was induced by photons, using \( H_G^{SE} = G^{AP} \times 3.21 / 1.17 \).

For thick films of DNA (43.6 µg, φ1.3 ± 0.2 mm), 96.3 % of X-rays were absorbed by DNA, while 3.7 % of X-rays passed through DNA, inducing SE emission from the tantalum surface. The mean X-ray-absorbed and SE-absorbed dose rates are 0.94 and 0.0027 Gy s\(^{-1}\), respectively. Thus the DNA damage was assumed to arise solely from X-rays.

For monolayer DNA (100 ng, φ6.0 ± 0.2 mm), 0.05 % of X-ray photons interacted with DNA, while 99.95 % of X-rays passed through DNA, interacting with the tantalum foil
and inducing SE emission from the tantalum surface. Assuming that all the energy of the interacting photons had been absorbed by the DNA, the mean X-ray-absorbed dose rate would have been 3.21 Gy s⁻¹. On the other hand, if all the energy of SE emitted from the tantalum surface had been absorbed by the DNA monolayer, the mean SE-absorbed dose rate would have corresponded to 1.17 Gy s⁻¹ \( [1.16 \times 10^8 \text{ electrons s}^{-1} \times 5.8 \text{ eV/electron} \times 1.6 \times 10^{-19} \text{ J/eV} / (1 \times 10^{-10} \text{ kg})] \). Thus, for monolayer DNA, the damage was induced by both X-ray photons and SEs emitted from tantalum. However, both of the above assumptions underestimate the G value for SEs. Since the interaction of Alkα X-rays with DNA is mainly via the photoelectric effect, it results in photoelectrons and Auger electrons, having electron energies of 960 and 520 eV\(^{24}\), and an axial penetration of about 18 and 8 nm\(^{16}\), respectively. For monolayer DNA, even if 0.05 % of X-rays interacted with DNA, most of the resulting photoelectrons and Auger electrons were transmitted through the 2 nm thickness of monolayer DNA and escaped into vacuum or tantalum. Furthermore SEs with kinetic energies over about 200 eV, which are also able to penetrate monolayer DNA and escape to vacuum, account for 0.3 % of the total electron yield but more than 28 % of the total energy of emitted SEs. Therefore, the \( L^{GSE} \) is the lowest limit of the G value for SE. On the other hand, the assumption that no damage in the monolayer was induced by photons overestimates the G value for SE, although this is partly compensated by the overestimation on the complete capture of SEs by monolayer DNA. Thus the \( H^{GSE} \) is a high but not highest possible limit of the G value for SE. The G's obtained in this study are in good agreement with the literature\(^{1,6,23}\). The quantum yields for DNA SSB and DSB by SE (average energy of 5.8 eV) were derived from \( L^{GSE} \) to be \( (4.8 \pm 0.3) \times 10^{-2} \) and \( (4.6 \pm 0.4) \times 10^{-3} \), respectively. These values are higher than
reported quantum yields for SSB and DSB by 10 eV mono-energetic electrons\textsuperscript{10} and SSB by 0.8 eV mono-energetic electrons\textsuperscript{14}, probably due to the different DNA composition and problems associated with the dosimetry of LEEs in the other studies. Whether SEs induce DNA CL is unclear at this point. The smaller $G^A$ than $G^P$ for DNA CLs shown in Table 1 is reasonable, because the duplex to duplex interactions in monolayer DNA is obviously less important than in a thick film. However, it is clear from the values in Table 1 that SEs are more efficient than X-ray photons to induce DNA SSB and DSB with an enhancement factor of at least 1.5 and 1.6, respectively. In other words, when the same amount of energy is deposited in DNA by photons and SEs, the latter produce more damage.

There are a number of mechanisms which can account for these differences, although the details can not be determined exactly at this stage. Al\textsubscript{K\alpha} X-rays interact with DNA mainly via the photoelectric effect and produce photoelectrons as well as Auger electrons. The absorption of electromagnetic radiation leads to the excitation (ca. 20 \%) and ionization (ca. 80 \%) of DNA.\textsuperscript{25} The fast-electrons from ionization react essentially via the emission of virtual photons\textsuperscript{26} so that both the initial photon interaction and the fast secondary electrons produce ions and excited vibrational and electronic states within DNA in similar proportions. X-rays also produce LEEs in the ionization process, but a large portion of the deposited energy flows into the creation of an ion or a hole, a mechanism which is practically absent in the case of LEE damage. More specifically, the studies of Razskazovskiy et al suggest that ionization of DNA, which takes a considerable amount of energy, gives rise to sugar centered radicals as precursors to SBs.\textsuperscript{6} Whereas X-rays and fast electrons produce electronic and vibrational excitation in
similar proportion, the relative abundance of electronic excitations may be larger for LEEs due to the formation of transient anions. Such a difference would result in a higher number of dissociative states produced per unit energy deposited by LEEs. In any case, due to polarization and the formation of transient anions, it takes less energy to break a chemical bound with a LEE than with a photon. The threshold energy for breaking a DNA strand with a photon lies around 7 eV\textsuperscript{27}, whereas a LEE induces SSB in DNA by dissociative electron attachment (DEA) via the formation of shape resonances located at 0.8 and 2.3 eV\textsuperscript{14}. Core-excited resonances induce SSB and DSB\textsuperscript{10-13} from 5 to 15 eV. These resonances consist of an electron temporarily captured by the positive electron affinity of an electronic excited state within DNA. The quantum yield of DNA SSBs at 0.8 eV is 15 fold that around 10 eV, suggesting that low-lying shape resonances in DNA are highly efficient for breaking the phosphodiester backbone. Thus, SEs emitted from tantalum with an energy distribution peaking around 1.4 eV can cause considerable DNA SBs via DEA.

**LEE enhancement factor (LEEEF) as a function of photon energy**

As the energy of X-rays increases, the attenuation in monolayer DNA decreases, such that the contribution of

**Figure 4** LEE enhancement factor (LEEEF) as a function of photon energies for SSB and DSB in monolayer DNA deposited on tantalum
SEs from the metal to the yield of products becomes larger. Considering only the dose imparted by the slow SEs emitted from the tantalum substrate, it is therefore instructive to define a LEE enhancement factor (LEEEF) for monolayer DNA to reflect this energy dependence. We define the LEEEF as the ratio of yield of products in monolayer DNA induced by LEE (slow SEs, $E_k \leq 10\text{eV}$) emission from Ta vs. that induced by photons. The LEEEF for 1.5 keV photons was derived to be at least 0.2 for both DNA SSB and DSB, by taking average $G$ values for SEs ($0.5^1G^{SE} + 0.5^0G^{SE}$). X-rays induce the emission of large amounts of SEs from metal surfaces, typically as a narrow peak below 10 eV for the low energy part and relatively sharper elastically scattered photoelectrons and Auger electrons for the high energy part. Although the increasing number of photoelectrons and Auger electrons at higher photon energies destroys the proportionality between total quantum yield ($\eta_b$) and $E\mu(E)^{28}$, the quantum yield for LEEs ($\eta_{ls}$, $E_k \leq 10\text{eV}$) was experimentally shown to vary approximately as $E\mu_{en}(E)/\rho^{17}$. Moreover, the energy spectrum for LEEs is independent of photon energy$^{21}$. Assuming that the $G$ values for SSB and DSB are independent of photon energy, we extrapolate the LEEEF for X-rays from 1.5 keV to 150 keV (i.e. to energies of medical diagnostic X-rays). As seen from Figure 4, the LEEEF varies with photon energy and is over 15 within the range of diagnostic X-ray energies.

In conclusion this first comparison study of DNA damage induced by X-rays and SEs under identical experimental conditions shows SEs to be more efficient in causing DNA SSB and DSB than X-ray photons; i.e. the $G$ values for SSB and DSB induction by SEs are at least 1.5 and 1.6, and could be as high as 4.3 and 4.4 times, of those by X-ray photons for the same damage, respectively. This study also shows that SE emission from
metal surfaces induced by ultrasoft X-rays (1.5 keV) may serve as an alternative to conventional electron guns to investigate the effects of SEs on biological media. The advantages of this method reside in its ability to determine G values for LEE-induced damage and to allow comparison of this value to that obtained with high energy photons under identical experimental conditions.

Acknowledgment. This work was supported by the Canadian Institutes of Health Research.

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Enhanced DNA damage induced by secondary electron emission from a tantalum surface exposed to clinical X-rays

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Number of figures: 3
Number of tables: 1

Proposed running head: Enhanced DNA damage by SE

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Both thick and thin films of pGEM®-3Zf(-) plasmid DNA deposited on a tantalum foil were exposed to soft X-rays (effective energy of 14.8 keV) for various times in air under relative humidities of 45 % (\(\Gamma \approx 6\), \(\Gamma\) is defined as the number of water molecules per nucleotide) and 84 % (\(\Gamma \approx 21\)), respectively. For a thin film of DNA, the damage was induced mainly by secondary electrons (SE) emitted from the tantalum. For a thick film, the DNA damage was induced chiefly by X-ray photons. Different forms of plasmid DNA were separated and quantified by agarose gel electrophoresis and laser scanning. The exposure curves for the formation of nicked circular (single strand break, SSB), linear (double strand break, DSB), and interduplex crosslink forms 1 and 2 (crosslinks, CL) were obtained for both thick and thin films of DNA, respectively. The SE enhancement factor for SSB, DSB and CL of the thin film of DNA were derived to be: 5.0 ± 0.8, 3.6 ± 1.0 and 29 ± 16 at \(\Gamma \approx 6\), and 8 ± 1, 9 ± 2 and 11 ± 3 at \(\Gamma \approx 21\), respectively. This study provides a molecular basis for understanding the enhanced biological effects at interfaces during X-ray diagnostic examination and radiotherapy.
INTRODUCTION

Radiation dose perturbation at low and high Z material interfaces has long been a subject of investigation in the fields of medical physics and radiation oncology. The dose enhancement at the tissue-metal interface has been confirmed by dose measurements\textsuperscript{1-3} and Monte Carlo calculations\textsuperscript{4-6}. Niroomand-rad et al reported a dose enhancement of about 20\% at the upper surface of the tissue-titanium dental implant interface in head and neck cancer patients treated with 6 MV and 10 MV photon beams\textsuperscript{1}. Head and neck cancer patients having surgery to remove tumors located in oropharynx and oral cavity often require mandible reconstruction. In addition, these patients usually need postoperative radiotherapy. Thermoluminescent dosimetry measurements by Allal et al showed marginally significant dose enhancement (6 ± 5\%) at bone/titanium interfaces around a titanium hollow-screw osseointegrating reconstruction plate using both \textsuperscript{60}Co and 6 MV photon beams\textsuperscript{2}. Melian et al reported a higher dose enhancement percentage, eg. 40\% at the Vitallium plate with \textsuperscript{60}Co\textsuperscript{3}. The enhancement factor was found to increase as the effective atomic number of the plate. Li et al reported a 5-10\% overdose at a depth of 0.5 mm in the metallic esophageal stents for 6 MV, 15 MV external photon beams and for a \textsuperscript{192}Ir brachytherapy source\textsuperscript{4}.

The corresponding enhanced biological effects at the interfaces of tissue and metals were also documented in the literature. Regulla et al reported dose enhancement up to more than a factor of 100 in tissue-equivalent polymethylmethacrylate close to a thin metallic gold foil using 40 to 120 kV heavily filtered X-rays\textsuperscript{7}. Correspondingly, enhanced interface biological effects, such as cell inactivation\textsuperscript{7}, the induction of dicentric chromosomes, centric rings and excess acentric fragments\textsuperscript{8}, as well as RBE and H ratio for cytogenetic effects\textsuperscript{9}, were observed. X-induced secondary electrons (SE) emission from gold surfaces was proposed to
account for this interface enhancement effect. Zellmer et al.\textsuperscript{10} and Rosengren et al.\textsuperscript{11} also reported an enhanced cell inactivation due to backscattered SE from metal surfaces induced by X-rays and $\gamma$-rays. A study of DNA damage induced by backscattered SE might provide additional insights into the molecular basis for the observed biological effects, however, such an investigation has never been reported.

Cellular DNA is mainly in the B-form and contains about 20 bound water molecules per nucleotide in the first ($\Gamma < 9$) and second ($9 \leq \Gamma \leq 20$) layers of hydration.\textsuperscript{12,13} The first layer consists of contiguous surface water while the second layer represents amorphous water. $\gamma$-radiolysis of water produces electrons and holes. Electrons readily transfer from both the first and second layers of water to DNA, but only holes of the first hydration layer can transfer to DNA while holes of the second hydration layer react with water to form hydroxyl radicals which subsequently react with DNA.\textsuperscript{14,15} Accordingly, hydration levels affect the yields of single and double strand breaks (SSB and DSB), unaltered nucleobase release and base damage in $\gamma$-irradiated DNA.\textsuperscript{16-19} Therefore, it is interesting to compare the influence of DNA conformation and hydration levels on the DNA damage induced by X-rays and SE.

In this study, both thick and thin films of pGEM\textsuperscript{®}-3Zf(−) plasmid DNA deposited on a tantalum foil were exposed to soft X-rays (effective energy of 14.8 keV) for various times in air under relative humidities of 45 % ($\Gamma \approx 6$, $\Gamma$ is defined as the number of water molecules per nucleotide) and 84 % ($\Gamma \approx 21$), which cause the DNA to adopt A and B conformations, respectively. Different forms of plasmid DNA were separated and quantified by agarose gel electrophoresis and laser scanning. The comparison between
the results from thick and thin films demonstrates that DNA damage is enhanced by SE and influenced by the hydration level.

**MATERIALS AND METHODS**

*DNA preparation and manipulation*

pGEM\textsuperscript{®}-3Zf(-) plasmid DNA (3197 bps, Promega) was extracted from E coli DH\textsubscript{50} and purified with the QIAfilter Plasmid Giga Kit (Qiagen). Agarose gel electrophoresis analysis showed that 95 % of the purified plasmid DNA was in the supercoiled form and the rest was in the concatemeric (4 %) and nicked circular (1 %) forms. The DNA pellet was redissolved in distilled and de-ionized water (dd H\textsubscript{2}O). Under a dry nitrogen atmosphere, either 51.6 µg in 3 µL of ddH\textsubscript{2}O or 25 ng of DNA in 5 µL of ddH\textsubscript{2}O was deposited on a chemically clean pre-frozen tantalum (Ta) sheet (−130 °C) which was fixed on an aluminum holder, then lyophilized with a hydrocarbon-free sorption pump under a pressure of 3 mtorrs for one hour. The 51.6 µg of lyophilized DNA formed a thick film (\sim 45 µm) with a diameter of 1.0 ± 0.1 mm, while 25 ng formed a thin film (\sim 15 nm) with a diameter of 1.2 ± 0.1 mm. Once removed from the vacuum, the lyophilized DNA on Ta was transferred to a sealed box (Fig. 1) and stored at 4 ± 1°C overnight. The humidity of the box was controlled with saturated aqueous solutions of either K\textsubscript{2}CO\textsubscript{3} or KCl to give 45 ± 3 % or 84 ± 3 %, corresponding to γ = 6 or 21 for DNA, respectively.\textsuperscript{20} DNA was kept inside the box at 4 ± 1 °C during the irradiation. The box was surrounded by ice to maintain the temperature of 4 ± 1 °C. After irradiation, DNA was immediately dissolved in cold 10 mM Tris-Cl, pH 7.5. The recovery rate was over 98 %.
**X-ray irradiation and dosimetry**

Figure 1 shows a photograph of the home-made box for X-ray irradiation. The 14.5 \( \times \) 19.0 \( \times \) 5 cm box is made of 10 mm thick aluminum. It has two valves on a wall, allowing the exchange of air to nitrogen or other atmosphere, and four Teflon columns (\( \phi \) 2.5 cm, height: 4.0 cm) on the bottom as sample supports. Each Teflon column is capped with aluminum, which has a hole at the center to secure the aluminum sample holder and has a connection to the ground. Its cover is made of 9.525 mm thick Plexiglas with four 2.54 cm diameter holes in a row and a 3 mm wide indented frame of 17.14 \( \times \) 4.45 cm around the holes. The four holes sit directly on top of four Teflon columns. A thin film of Mylar (6\( \mu \) thick, SPEX CertiPrep, US) is fixed on the frame with a rubber ring to seal the box but allows more than 99.99% transmission of X-rays. A pool of aqueous solution inside the sealed box controls the relative humidity, which is read with a digital thermo-hygrometer (VWR).

Soft X-rays were generated with a superficial therapy X-ray unit (PANTAK Therapax 3 series) at Université de Sherbrooke Hospital Center, Sherbrooke, Quebec, Canada. A 0.4 mm Al filter, a potential energy of 30 kVp and a filament current of 7.6 mA were selected, resulting in an X-ray beam with an effective energy of 14.8 keV\(^{21}\). A treatment cylinder (\( \phi \) 2.5 cm, 15 cm source to surface distance (SSD)) was used to expose hydrated DNA and radiochromatic films to X-rays. The absorbed dose rate in water (\( D_S^w \)) at the surface of sample holder was 0.035 Gy s\(^{-1}\), measured by GAFCHROMIC\(^{\circledR}\) HD-810 radiochromatic films\(^{22}\). Radiochromatic films also showed the homogeneous distribution of X-rays within the cylinder area. The incident fluence rate of photons (\( \phi_0 = 1.1 \times 10^{10} \) photons s\(^{-1}\) cm\(^{-2}\)) was calculated from \( D_S^w \) using eq 1\(^{22}\):
\[ \phi_0 - A D_S^w / [(\mu_{en/\rho}) w E] \] (1)

where \( A \) is a conversion coefficient of \( 6.24 \times 10^{15} \text{ eV g}^{-1} \text{ Gy}^{-1} \), \( (\mu_{en/\rho})_w \) is the mass energy absorption coefficient of water of \( 1.37 \text{ cm}^2 \text{ g}^{-1} \) at 14.8 keV and \( E \) is the photon energy of 14800 eV.

The mean X-ray absorbed dose rate was calculated with eq 2:

\[ \bar{D}_m = \phi_0 (\mu_{en/\rho}) E \{1 - \exp[-(\mu/\rho)d]\} / [A(\mu/\rho)d] \] (2)

where \( d \) is the sample density in g cm\(^{-2} \); \( \mu_{en/\rho} \) and \( \mu/\rho \) are the mass energy absorption coefficient and the mass attenuation coefficient of the sample in cm\(^2 \) g\(^{-1} \), respectively.

The sodium salt of pGEM\(^\text{®}\)-3Zf(-) plasmid DNA was used in this study. With 14.8 keV X-rays, \( \mu_{en/\rho} \) and \( \mu/\rho \) for the DNA are calculated based on the faction by weight and the coefficient for each component element to be 2.20 and 2.51 cm\(^{-2} \) g\(^{-1} \) for \( \Gamma = 0 \), 2.00 and 2.31 cm\(^{-2} \) g\(^{-1} \) for \( \Gamma = 6 \), and 1.67 and 1.97 cm\(^{-2} \) g\(^{-1} \) for \( \Gamma = 21 \), respectively\(^\text{23} \). Thus, the mean X-ray absorbed dose rates in thin films of DNA are 0.0563, 0.0512 and 0.0450 Gy s\(^{-1} \) for \( \Gamma = 0 \), 6 and 21, respectively. The mean X-ray absorbed dose rates in thick films of DNA are 0.056, 0.0507 and 0.0443 Gy s\(^{-1} \) for \( \Gamma = 0 \), 6 and 21, respectively.

**X-ray induced secondary electron emission from the tantalum surface**

The X-ray induced SE emission from the tantalum surface was measured using a Keithley 610C solid state electrometer connected between the tantalum target (\( \phi \) 2.5 cm, 4.9 cm\(^2 \)) and ground. The mean energy of SE was determined using GAFCHROMIC\(^\text{®}\) radiochromatic films, consisting of an active layer (21.7 \( \mu \)m, 1.08 g cm\(^{-3} \)) and a polyester base (99 \( \mu \)m, 1.35 g cm\(^{-3} \)), but no-top layer. The mean X-ray absorbed dose rate in the active layer was calculated with Eq 2 to be 0.0195 Gy s\(^{-1} \). For the active layer, \( d \) was
2.34×10⁻³ g cm⁻², $\mu_{\text{cm}}/\rho$ and $\mu/\rho$ were 0.759 and 0.103 cm² g⁻¹ ²², ²³. The attenuation of X-rays by the polyester base and the active layer was 1.50 % and 0.24%, respectively. Radiochromatic films placed on Ta with either the active layer or the polyester base facing the Ta were exposed to X-rays for various times. Following 24 hr storage in the dark, the films were scanned using an HP ScanJet 6100C colour scanner, which was driven by HP Precision Scan Pro 1.01. Auto exposure and sharpen level were turned off. The intelligent scanning technology for best quality scaling, best quality sharpening, maximum pixel depth and best quality scan speed was also turned off from the preference menu. The output resolution was set at 150 dpi. The output type was set as ‘true colour’. The colour saturation was kept constant at 100%. The colour hue was set as $x = -100, y = 0$. The scanned images were processed with Corel Picture Paint 7. The average pixels ($s$) for the red channel were read from the histogram. The net optical density (absorbance, ABS) was defined as $\log_{10}(s_0/s)$, where $s_0$ is the average pixel of an unexposed film ²².

*Agarose gel electrophoresis*

Six forms of double stranded DNA were separated by 1 % agarose gel electrophoresis run in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 6.7 V cm⁻¹ for 7 minutes and 5.0 V cm⁻¹ for 75 minutes. These forms are: crosslinked form I(C-C), crosslinked form 2(S-C), circular (C), linear, concatemeric (CM) and supercoiled (S) DNA, in order of increasing migration rate ²⁴. Both gel and DNA were prestained with SYBR® Green I (Molecular Probes) (1X for gel and 20X for DNA, respectively). About 125 ng of DNA was loaded into each well. After electrophoresis, the gel was scanned with STORM860 Blue Fluorescence (Molecular Dynamics) at an excitation wavelength of 450 nm. The relative amount of DNA in each form was quantified with ImageQuant.
(Molecular Dynamics). A factor of 1.7 obtained from previous studies was used to correct for the weaker binding of SYBR® Green I to supercoiled DNA compared to nicked circular and linear configurations\(^{24}\).

*Calculation of G values*

The absolute amount of plasmid DNA in all forms was determined by measuring its UV absorption at 260 nm, based on that a solution with an optical density of 1 at 260 nm contains 50μg of DNA per millimetre\(^{25}\). The relative amount of different forms of plasmid DNA was obtained from agarose gel electrophoresis.

The apparent G values (\(G^A\) in mol J\(^{-1}\)) for the induction of DNA SSB (C), DSB (L), crosslink 1 (S-C,\(_1\)) and crosslink 2 (C-C) by X-rays were derived from the initial linear slopes (A in % kGy\(^{-1}\)) of the respective dose response curves given by\(^{26}\):

\[G^A = \frac{A}{(f \times MW)} \tag{4}\]

where MW is the molecular weight of pGEM\(^{®}\)-3Zi(-) plasmid DNA sodium salt (2115979, 2807107 and 4534926 Da for hydrated DNA of \(\Gamma = 0, 6\) and 21, respectively) for the formation of SSB and DSB and the loss of S. For the formation of DNA interduplex crosslinks (CL), MW is the molecular weight of the DNA dimer (4231958, 5614214 and 9069851 Da for hydrated DNA of \(\Gamma = 0, 6\) and 21, respectively). \(f\) is the percentage of S in control DNA.

**RESULTS AND DISCUSSION**

*X-ray Induced SE from Tantalum: Yield and Energy*

The current of the X ray-induced SE from the tantalum surface (φ 2.5 cm, 4.9 cm\(^2\)) was 0.20 ± 0.02 nA, giving an electron current density of \((2.6 \pm 0.3) \times 10^6\) electrons cm\(^{-2}\) s\(^{-1}\).
Thus, the quantum yield of X-ray induced electron emission is 0.024 ± 0.003 electron photon⁻¹, in good agreement with the value reported by Seguin et al (0.03 ± 0.01 electron photon⁻¹)²⁷. Dolan reported that the quantum yield for fast SE (ηf) emitted from 25 µm thick tantalum was 0.0116 ± 0.0017 and 0.0085 ± 0.0012 electron photon⁻¹ for 50 and 100 kV X-rays (effective energies of 30 and 54 keV), respectively²⁸. ηf varied approximately as E²¹µen (E)/ρ, as proposed by Henke et al²⁹. Here, E is the photon energy and µen(E)/ρ the mass absorption coefficient of tantalum. Thus, for 14.8 keV X-rays, ηf was estimated as 0.015 electron/photon, giving a quantum yield of 0.009 electron/photon for slower SE.

The mean energy of SE was determined using GAFCHROMIC® radiochromatic films with no-top layer. Radiochromatic films on Ta with either the active layer or the polyester base facing the Ta were exposed to X-rays for 2.5-10 min. After 24 hr storage in dark, the optical density of the films was quantified. Figure 2 shows the linear dependence of ABS on the mean X-absorbed dose in the active layer for both placements of the film on Ta. The linear least square fits of ABS to the absorbed dose give slopes of 0.0378 ± 0.0002 and 0.080 ± 0.002 Gy⁻¹ for the polyester base and the active layer facing the Ta, respectively. The X-ray-induced SE from the tantalum surface is responsible for the higher slope when the active layer rather than the polyester base is facing the Ta. The radiochromatic film is composed of an active layer (21.7 µm) and a polyester base (99 µm). When the polyester base was put directly on the Ta, all SE emitted from the Ta was absorbed by the polyester and could not reach the active layer, thus, the optical absorbance merely resulted from X-rays absorbed in the active layer. When the active layer sat directly on the Ta, all SE from the Ta were absorbed by the active layer, and
were responsible for the increase of the slope. Thus the mean absorbed dose of SE in the active layer was derived to be 0.0217 Gy s\(^{-1}\) and the mean energy of SE to be 1.22 keV.

For thick films of DNA, the thickness of the DNA was 8.72 and 14.09 mg cm\(^{-2}\) for hydrated DNA of \(\Gamma = 6\) and 21, respectively. Thus, all SE were captured by the hydrated DNA. The mean absorbed dose rate of SE in thick film DNA was derived to be 5.70 and 3.53 mGy s\(^{-1}\) for the hydrated DNA of \(\Gamma = 6\) and 21, respectively.

The energy spectra of the SE typically exhibit a narrow peak below 100 eV for the low energy part \(^{22,26}\) (peak at 1.4 eV) and relatively sharper elastically scattered photoelectron and Auger electron peaks at high energies. For thin films of DNA, the thickness of the DNA was 2.93 and 4.74 \(\mu\)g cm\(^{-2}\) for hydrated DNA of \(\Gamma = 6\) and 21, respectively. According to the calculations of LaVerne and Pimblott\(^{30}\), the inelastic mean free path of 5, 10 and 20 keV electrons in DNA is 1.52, 2.70 and 4.79 \(\mu\)g cm\(^{-2}\), respectively and the most probable energy loss for an electron is 23 eV. Thus, the fast SE with \(E_k > 5\) keV should deposit only small fraction of their energy in the DNA before escaping the film. SE escaping the film may react with \(O_2\) in the box to form \(O_2^-\); however, the rate constants of \(O_2^-\) reacting with DNA is less than \(10^6\) mol\(^{-1}\) dm\(^3\) s\(^{-1}\) \(^{31}\). Moreover, \(O_2^-\) is known to cause oxidative DNA damage via the combination of \(O_2^-\) with guanine neutral radicals\(^{32}\). Thus, the possibility of the induction of DNA SB and CL by \(O_2^-\) is assumed to be negligible in this study.

*Dependence of DNA SSB, DSB, and CL formation on Absorbed Dose*

107
Figure 3 shows the dependence of the loss of S and CM as well as the formation of C, L, C-C and S-C in both thick and thin films of DNA \((\Gamma = 6)\) on the mean X-ray-absorbed dose in hydrated DNA. Similar exposure curves were also obtained for \(\Gamma = 21\). The larger slope for thin compared to thick films suggests an enhancement of DNA damage due to SE emission from Ta. The apparent G values \((G^{AP})\) for DNA SSB, DSB and CL were derived from the slope of linear least square fits of the respective exposure curves for the formation of C, L, C-C and S-C, and compiled in Table 1. Table 1 also shows the derived G values for X-ray photons, and compares the enhancement factor of SE vs photons to cause DNA SSB, DSB and CL in thin films of DNA.

**Table 1** G values (in nmol J\(^{-1}\)) for the formation of different forms of DNA induced by X-ray photons and secondary electron enhancement factor (SEEF) for thin film of DNA at two DNA hydration levels

<table>
<thead>
<tr>
<th></th>
<th>thick film DNA, (G^{AP})</th>
<th>thin film DNA, (G^{AP})</th>
<th>X-photon, (G^p)</th>
<th>SEEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circular (SSB)</td>
<td>90 ± 8 (109 ± 9)</td>
<td>482 ± 28</td>
<td>81 ± 7 (96 ± 8)</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td>Linear (DSB)</td>
<td>5.6 ± 0.8 (7 ± 1)</td>
<td>23 ± 2</td>
<td>5.1 ± 0.7 (6.0 ± 0.9)</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>C-C (CL1)</td>
<td>1.0 ± 0.5 (1.2 ± 0.6)</td>
<td>19 ± 1</td>
<td>0.9 ± 0.4 (1.1 ± 0.5)</td>
<td>26 ± 14</td>
</tr>
</tbody>
</table>

\(^a\) G values are based on the entire sample mass (DNA + hydration water), except those shown in parentheses, which are based on the mass of dry DNA. \(G^{AP}\) assumes all the energy of the photons interacting with DNA was imparted to the DNA and no damage was induced by SE emitted from Ta. \(G^p\) was derived from the data of thick film DNA, using \(G^p = G^{AP} \times \hat{D}_m^X / \left(\hat{D}_m^X + \hat{D}_m^{SE}\right)\) and assuming that all the energy of the absorbed photons and the SE emitted from Ta was imparted to the DNA and the efficiency of X-photon and SE damage to DNA was the same. The SEEF was calculated from the expression \([G^{AP}\text{(thin)} - G^p]/G^p\).
G^{AP} assumes all the energy of the photons interacting with DNA was imparted to the DNA and no damage was induced by SE emitted from Ta. Actually both X-ray photons and SE (i.e., G^{real}) from Ta contribute to the damage in both thick and thin films of DNA. For a thick film the mean absorbed dose rates for X-ray photon and SE (\(\dot{D}_m^X\) and \(\dot{D}_m^{SE}\)) are 50.7 and 5.70 mGy s\(^{-1}\) for \(\Gamma = 6\) and 44.3 and 3.53 mGy s\(^{-1}\) for \(\Gamma = 21\), respectively. Thus, the damage in the thick film is induced chiefly by X-ray photons. Considering that:

\[
G^{AP}(\text{thick}) = \frac{\text{Amount of damage}}{\text{Absorbed Energy of photons}}
\]

\[
G^{real}(\text{thick}) = \frac{\text{Amount of damage}}{\text{(Absorbed Energy of photons} + \text{Absorbed Energy of SE))}}
\]

we have in a first approximation

\[
G^{real}(\text{thick}) \backsimeq G^P
\]

\[
= G^{AP}(\text{thick}) \times \frac{\text{Absorbed Energy of photons}}{\text{(Absorbed Energy of photons} + \text{Absorbed Energy of SE))}}
\]

\[
= G^{AP} \times \frac{\dot{D}_m^X}{(\dot{D}_m^X + \dot{D}_m^{SE})}
\]
As seen in Table 1, each value of $G^{AP}(\text{thin})$ is significantly larger than that of $G^p$ for the same type of damage and hydration level. Thus for a thin film of DNA the damage was induced mainly by SE emitted from the tantalum. We define a SE enhancement factor (SEEF) for the thin film of DNA as the ratio of yield of products in the thin film induced by SE emitted from Ta vs that induced by photons.

\[ \text{SEEF} = \frac{\text{Density of damage for thin film} - \text{Density of damage for thick film}}{\text{Density of damage for thick film}} \]

\[ = \frac{G^{AP}(\text{thin}) - G^p}{G^p} \]

Based on the mass of the entire DNA sample, the $G^p$ values for DNA SSB and DSB are smaller for $\Gamma=21$ than for $\Gamma=6$ while those of CL are similar for both $\Gamma$. However, based on the mass of dry DNA, the $G^p$ values of DNA SSB and CL are slightly higher for $\Gamma=21$ than for $\Gamma=6$, while those of DSB are similar for both $\Gamma$. The $G^p$ values of DNA SSB, DSB and CL are all higher than those obtained under vacuum ($\Gamma=2.5$)\textsuperscript{26}. These results suggest that both layers of bound water contribute to DNA damage, but their efficiency is lower than direct excitation and ionization of DNA. Yokoya \textit{et al.} reported the effects of hydration on the induction of SSB and DSB in plasmid DNA films by $\gamma$-radiation in air\textsuperscript{16}. They calculated $G$ values based on the mass of dry DNA and observed a small increase in $G$ values for SSB and DSB with hydration, eg. $G$(SSB) increases from $65 \pm 8$ to $71 \pm 6$ nmol J\textsuperscript{-1}, and $G$(DSB) from $5.4 \pm 0.7$ to $5.9 \pm 0.5$ nmol J\textsuperscript{-1} when $\Gamma$ increases from $8 \pm 2.2$ to $24.5 \pm 6.9$. No induction of CL was mentioned in their report. It is worth noting that the DNA samples used in the present study did not contain any salts (other than the sodium counter ions), but in Yokoya \textit{et al.}'s study the DNA samples contained Tris. Tris
II. RESULTS

is an e' and 'OH scavenger. In our study, the ratios of G(SSB)/G(DSB) (16 and 18 for Γ =
6 and 21, respectively.) is much higher than theirs (12). It is not clear whether Tris is
responsible for these differences.

These results can be understood by considering the following reactions\textsuperscript{16,19} induced by
X-rays, electron and hole transfer from hydration water to DNA and within DNA, and the
dependence of DNA conformation on hydration levels.

\begin{equation}
\text{DNA} \rightarrow e^- + (\text{DNA})_{d}{}^{*+}
\end{equation}

\begin{equation}
\text{H}_2\text{O} \rightarrow e^- + \text{H}_2\text{O}^{*+}
\end{equation}

\begin{equation}
\text{H}_2\text{O}^{*+} + \text{DNA} \rightarrow (\text{DNA})_{w}{}^{*+} + \text{H}_2\text{O}
\end{equation}

\begin{equation}
\text{H}_2\text{O}^{*+} + \text{H}_2\text{O} \rightarrow \cdot\text{OH} + \text{H}_3\text{O}^+
\end{equation}

\begin{equation}
e^- + \text{DNA} \rightarrow \text{DNA}^-
\end{equation}

\begin{equation}
e^- + \text{H}_2\text{O} \rightarrow (\text{H}_2\text{O})^- \rightarrow \text{H}^- + \cdot\text{OH}
\end{equation}

\begin{equation}
e^- + n\text{H}_2\text{O} \rightarrow e_{\text{aq}}^-
\end{equation}

\begin{equation}
\cdot\text{OH} + \text{DNA} \rightarrow \text{DNA}^-
\end{equation}

\begin{equation}
e^- + (\text{DNA})_{d/w}{}^{*+} \rightarrow \text{DNA}
\end{equation}

\begin{equation}
(\text{DNA})_{d}{}^{*+}, \text{DNA}^- \text{ and DNA}^* \rightarrow \text{SSB}, \text{DSB, base lesions, CL}
\end{equation}

\begin{equation}
(\text{DNA})_{w}{}^{*+}, e_{\text{aq}}^- \rightarrow \text{base lesions}
\end{equation}
Electrons from hydration layers within $\Gamma = 21$ and all $H_2O^+$ within $\Gamma = 9-11$ readily transfer to DNA, while part of $H_2O^+$ from $\Gamma = 12$ to 14 and all $H_2O^+$ from $\Gamma > 15-20$ is converted to $^\cdot OH$ $^{12,13}$. Dry $e^-$ transferring from hydration layers to DNA, might either lead to additional SSB and DSB via dissociative electron attachment (DEA)$^{33-35}$ or protect DNA from SB via the recombination with DNA holes (reaction 12). Hole transfer from hydration layers to DNA is assumed to mainly cause base lesions rather than SB and CL. But $^\cdot OH$, diffusing from the hydration layer to DNA, cause both SB and CL. Higher hydration levels of DNA were reported to accelerate the protonation of DNA radical anions and deprotonation of DNA radical cations$^{36}$, but to weaken interduplex electron and hole tunneling$^{20}$. When based on the mass of dry DNA, the increase of $G^p$ values of DNA SSB with $\Gamma$ can be explained by attacks of $e^-$ and $^\cdot OH$ on DNA, as well as higher reactivity of radical ion pairs to form products at higher hydration. Since DSB is resulted from a single event, the reaction of DNA with $^\cdot OH$ mainly causes additional SSB rather than DSB. The $G^p$ values of DNA DSB hardly change when $\Gamma$ increases from 6 to 21. The $G^p$ value of SSB for dry DNA is 57 nmol J$^{-1}$ $^{24}$, while the $G$ value of SSB for aerated aqueous solution of the same plasmid DNA at pH 7.8 was determined to be 50 nmol J$^{-1}$ $^{40}$. This suggests that direction ionization is more efficient to induce DNA SSB than $^\cdot OH$. On the other hand, part of $H_2O^+$ from $\Gamma > 15-20$ transfer to DNA, only causing base damage not SB. Therefore, when based on the mass of the entire DNA sample (DNA + water), $G^p$ values of DNA SSB and DSB decrease as $\Gamma$ increases from 6 to 21.
In contrast to $G^p$, the $G^{Ap}$ values of DNA SSB and DSB in thin films are higher for $\Gamma=21$ than $\Gamma=6$. As $\Gamma$ increases, so does the thickness of a thin DNA film and the deposited energy of the SE in DNA. Thus, the dose enhancement increases. On the other hand, the density functional theory studies of electron interaction with DNA predicted that solvation should increase the yield of DNA SSB induced by low energy SE\textsuperscript{37,38}. The $G^{Ap}$ value of DNA CL in the thin film decreases as $\Gamma$. For $\Gamma=21$, DNA assumes a predominantly B-conformer, while for $\Gamma=6$, DNA is mainly in the A-form\textsuperscript{13}. This confirms our previous finding that DNA conformation plays an important role in the induction of CL and that the A-form DNA is more favorable to undergo DNA interduplex CL than the B-form\textsuperscript{24}. As a result, the SEEF of DNA SSB and DSB increases, while the SEEF of DNA CL decreases with $\Gamma$. The $G^{Ap}$ values of DNA SSB and DSB in thin films obtained in this study are significantly higher than those in monolayer film of DNA induced by ultrasoft X-rays under vacuum\textsuperscript{24}. In this study, we used higher X-ray photon energy (15 keV vs 1.5 keV), thicker thin film of DNA (~20-35 nm vs. 2 nm), hydrated DNA ($\Gamma=6$, 21) in air rather than dry DNA ($\Gamma=2.5$) under vacuum. Especially, the average energy of SE induced by 15 keV photons is 1.22 keV, much higher than 5.8 eV, the average energy of SE induced by 1.5 keV photons\textsuperscript{24}. That backscattered SE impart more energy to the thin film of this study than monolayer film of the previous study may be the main factor for the higher $G^{Ap}$ values obtained in this study.

The SEEF of SSB, DSB and CL in thin DNA with two hydration layers ($\Gamma=21$), similar to those in cells, is about 10. DSB and CL are highly toxic and mutagenic. The SEEF of DSB and CL may serve as a molecular basis for the observed enhanced cell inactivation\textsuperscript{7,10,11}, induction of dicentric chromosomes, centric rings and excess acentric
fragment due to SE emission from gold\textsuperscript{8}, and for the radiotherapy enhancement in mice using gold nanoparticles and X-rays\textsuperscript{39}.

In conclusion, this study shows that X-induced SE emission from tantalum results in a significant SEEFe for DNA SSB, DSB and CL. The hydration level of DNA has an effect on the SEEFe. This study provides a molecular basis for understanding the enhanced interface biological effects during X-ray medical diagnosis and radiotherapy.

ACKNOWLEDGMENTS

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Figure 1 A photograph of the home-made box used for X-ray irradiation.
Figure 2 Dependence of the net optical density of no top layer radiochromatic films on the mean X-ray absorbed dose in the active layer: the effect of X-ray induced electron emission from tantalum. Layer facing Ta: active layer, ○; polyester base: ▲. The error bars show the standard deviation of the ABS resulting from the SD of the pixel determination.
Figure 3 X-ray exposure curves for the formation of circular (a), linear (b), crosslink 1 (c) and 2 (d) DNA as well as the loss of supercoiled (e) and concatemeric (f) DNA in thick (○) and thin (▲) films of DNA. The points represent the mean of 3 independent experiments and the error bars represent the ± 1 standard deviation of the mean.
Induction of strand breaks by low energy electrons (8-68 eV) in a self assembled monolayer of oligonucleotides: effective cross sections and attenuation lengths

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Self-assembled monolayers (SAM) of a 5'-32P-labelled 3'-thiolated oligonucleotides chemisorbed on gold were bombarded by low energy electrons (LEE) of 8 to 68 eV. Shorter 5'-32P-oligonucleotides produced by LEE-induced strand breaks were separated with denaturing polyacrylamide gel electrophoresis and quantified by phosphor imaging. The yields of short oligonucleotides (γ) decrease exponentially with their length (n), following the equation \( \gamma = a \times e^{-bn} \), where a and b are constants, which are related to the average effective cross section per nucleotide for DNA strand break \( (\sigma_{\text{eff}}) \) and the attenuation length \( (AL = 1/b) \) of LEE, respectively. The AL decreases with LEE energies from 2.5 ± 0.6 nm at 8 eV to 0.8 ± 0.1 nm at 68 eV, whereas \( \sigma_{\text{eff}} \) increases from \( (3 \pm 1) \times 10^{-18} \) to \( (5.1 \pm 1.6) \times 10^{-17} \) cm² within the same energy range. The energy dependence of \( \sigma_{\text{eff}} \) shows a resonance peak of \( (2.8 \pm 0.9) \times 10^{-17} \) cm² at 18 eV superimposed on a monotonically rising curve. Transient electron attachment to the sugar phosphate backbone followed by decay into dipolar dissociation is proposed to account for this maximum.

I. Introduction

Ionizing radiation generates large quantities of low energy electrons (LEE) in cells (~ 40,000 are produced by a 1 MeV electron) with most of the distribution lying below 70 eV\(^1\). It has been demonstrated that LEE can induce strand breaks (SB)\(^2\text{-}^5\), base

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fragmentation\textsuperscript{6} and base release\textsuperscript{7} in dry DNA under vacuum. Depending on the energy, the mechanisms may involve resonance decay into dissociative states\textsuperscript{2,4}, dissociative electron attachment (DEA)\textsuperscript{8} via shape \textsuperscript{5,9} or core excited resonances\textsuperscript{4}, and direct or resonance dipolar dissociation (DD)\textsuperscript{10}. It has been also demonstrated that LEE are more efficient than X-ray photons for producing DNA SB.\textsuperscript{11} Knowledge of inelastic mean free paths (IMFP) or attenuation lengths (AL)\textsuperscript{12} for scattering of LEE within DNA, as well as the effective cross sections for LEE- induced DNA damage is of considerable importance to radiation biology, and is also necessary for predicting DNA damage in cells induced by ionizing radiation using Monte Carlo simulations\textsuperscript{13}.

Determination of IMFP, AL and scattering cross sections in solid matter are hampered with a number of difficulties which are related to the measurement of the absolute values of the parameters of the experiment. When extracting AL or cross sections from electron scattering experiments on thin molecular films, by far the most difficult parameter to determine and control, is the film thickness and its variation along the plane of the supporting substrate. This problem is particularly acute in the case of vacuum-dried DNA films which have been used to measure LEE-induced damage\textsuperscript{2-5,8}. In this case, clustering of the DNA molecules induces variation in the thickness of the film, which translates into considerable errors in the determination of the cross sections for SB by LEE impact\textsuperscript{3}. To virtually eliminate this major source of error, we propose the use of self-assembled monolayers (SAM) in LEE scattering experiments. In such a layer the molecules are uniformly oriented with a regular density over the substrate to which they are chemisorbed. Since the molecules can be chosen with all the same length, these characteristics produce a film of a precise thickness on the substrate. Here, we report the
result of measurements of SB induced by LEE incident on SAM of a DNA oligomer chemisorbed on gold\textsuperscript{14}. By analyzing our data with DNA sequencing procedures, we determine cross sections and AL for SB induction in a single strand of DNA. Whereas with previous experiments it has only been possible to obtain effective cross sections for a film of large biomolecules of a given thickness, we show that with the SAM technique an average cross section per scattering site can be extracted from the measurements.

II. Experiment

A 50-base long thiolated oligonucleotide (OligoS), 5'-\((\text{GCTA})_{12}\text{GC(CH}_2\text{)}_3\text{-SS-(CH}_2\text{)}_3\text{-OH-3'}\) (Proligo, HPLC purified) was labelled at the 5'-end with $^{32}\text{P}$. Two hundred and fifty pmol of OligoS was mixed with 0.37 MBq of $[^{32}\text{P}]\text{ATP}$ and 10 U of T4 Polynucleotide kinase (usb\textsuperscript{®}) in 25 μL of 1X One-Phor-All buffer plus (usb\textsuperscript{®}), and was incubated at 37 °C for 45 min, then at 75 °C for 10 min to inactivate the kinase. To remove free $[^{32}\text{P}]\text{ATP}$ and kinase, the reaction solution was passed on a Sephadex G-50 microcolumn which had been pre-equilibrated with 0.4 M phosphate buffer (pH 7.2). The recovery of OligoS was 47.4 ± 0.6 %, based on the radioactivity of a purified $^{32}\text{P}$-labelled OligoS before and after passing a similar Sephadex G-50 microcolumn. The radioactivity was measured with a biodegradable counting scintillant (Amershan Bioscience) and a 1214 RACKBETA liquid scintillation counter (LKB Wallac).

Gold slides (12mm × 12mm, Gold Arrandee\textsuperscript{™}) were rinsed with acetone, 95% ethanol and distilled and deionized water (ddH\textsubscript{2}O) and subsequently placed in an ultraviolet ozone cleaner for 30 min. This cleaning procedure was repeated once. Then each clean gold slide was immediately covered with 5 μL of the above $^{32}\text{P}$-labeled
OligoS, and was kept overnight in a sealed Petri dish at room temperature and 100% relative humidity. The reaction of OligoS with gold results in strong chemical bonds between 3'-S and gold substrate and a well oriented SAM of OligoS on gold, as shown by the group of Naaman\textsuperscript{14} (Fig.1). The radioactivity of OligoS measured before and after overnight incubation shows that 55 ± 7 % of OligoS attached to the gold surface with a density of (5.8 ± 0.5) \times 10^{12} \text{ molecules cm}^{-2}.

After deposition, the slide was subsequently immersed three times in 10 mL of 0.4 M phosphate buffer (pH 7.2), three times in 10 mL of sterile dd H\textsubscript{2}O for 20 minutes each, thoroughly rinsed with dd H\textsubscript{2}O, and dried in air, then immediately delivered to an ultrahigh vacuum (UHV) chamber. After the vacuum reached 5 \times 10^{-8} \text{ torr} (about 2 hours), the slide was exposed to an electron beam at room temperature. To obtain an exposure response curve, the target was exposed to 58 eV electrons with a constant incident current of 50 ± 2 n\text{A} for 3-10 min. The incidence of the electron beam was perpendicular to the gold surface. The energy resolution of the beam was 0.5 eV full width at half-maximum. The electron beam was adjusted to be homogeneous within a diameter of 5.5 ± 0.2 mm. The details of the apparatus and electron bombardment procedure have been described elsewhere\textsuperscript{3}. Here, at a constant incident electron current density (J) of (1.0 ± 0.1) \times 10^{12} \text{ electron cm}^{-2} \text{ s}^{-1}, eight targets were exposed for 10 min to the electron beams. The targets were bombarded with the electron beam set at 8, 12, 18, 28, 38, 48, 58 and 68 eV. The electron energy was determined by measuring the potential corresponding to the onset of the electron current transmitted through the SAM and collected at the gold substrate. Any potential difference due to charging of the sample was measured by a Kelvin probe. Such measurements after bombardment revealed that
the SAM did not charge, even at the maximum exposure of the present experiments. Targets that were not exposed to the LEE beam contained 2 ± 1% of random SB of OligoS and the yield of individual 5'-OligoS-F was independent of the length. Thus, we consider that 2 ± 1% of the yield arise from sample manipulation.

Once removed from the UHV chamber, the gold slide was covered with 30 µL of sterile ddH₂O to dissolve and collect 5'-oligonucleotide fragments (5'-OligoS-F) resulting from LEE-induced SB. This procedure was repeated three times. 95 ± 2 % of 5'-OligoS-F was recovered in 77 ± 3 µL of ddH₂O. 2.0 µL of the 5'-OligoS-F solution were used for radioactivity measurements, to derive the total yield of LEE-induced 5'-OligoS-F, while the rest was concentrated using a Speed Vac Concentrator (SAVANT). Fragments from concentrated 5'-OligoS-F were separated by electrophoresis using a 7 M urea denaturing 20% polyacrylamide gel (20×25cm) run at a constant power of 20 Watts for 60 min. A storage phosphor screen was exposed to the gel overnight at -20°C and subsequently scanned with a 633 nm laser using a STORM860 system in the phosphorescence mode (Molecular Dynamics). The gel was quantified using ImageQuant software (Molecular Dynamics). Molecular weight ladders were generated by random depurination using formic acid at room temperature followed by cleavage at apurinic sites with piperidine at 90°C to calibrate the length of 5'-OligoS-F. The number of each fragment was determined by integrating the area under each peak of the scan.

III. Results and discussion

Fig. 2 shows an exposure-response curve recorded for the total number of SB induced by 58 eV electrons. Linear least square fit to the experimental data through zero
gives a slope (quantum yield of SB) of \((9.1 \pm 0.3) \times 10^{-4}\) fragment electron\(^{-1}\). It provides a value of quantum yields in good agreement with those for single strand break (SSB) formation in plasmid DNA induced by LEE\(^3\).

The linear dependence of the total yield of LEE-induced 5'-OligoS-F on the number of incident electrons indicates that the products are formed as the result of a single event and that SAM of our OligoS do not charge when the total number of the incident electrons is less than \(3 \times 10^{14}\), as verified by surface potential measurements. After subtracting the background from sample manipulation, the yield \((y, \text{number of fragments})\) of LEE-induced 5'-OligoS-F is found to decrease with increasing length \((n, \text{number of nucleotide})\), following equation 1 for LEE of 8 to 68 eV:

\[
y(n) = a \times e^{-bn}
\]  

where \(a\) and \(b\) are constants. Figure 3 shows examples of the exponential decrease of peak area as a function of the length of OligoS-F for LEE energies of 8, 28 and 68 eV. A similar dependence was observed for LEE energies of 12, 18, 38, 48 and 58 eV. The sensitivity of the present experiment did not allow SB detection without larger errors at lower energies. Within the sensitivity of our experiments, no significant base preference for SB was observed for all energies, suggesting that the mechanism for inducing SB is fairly independent of the nature of the bases or it operates on the sugar phosphate backbone rather than the DNA bases. As shown in the denaturing polyacrylamide gel (Fig. 4), the main band of each OligoS-F co-migrated with its corresponding 5'-end fragments obtained by G + A chemical sequencing reactions, but was accompanied by a faster migrating band, similar to phosphoryl glycolate observed in g-ray induced deoxyribonucleic acid SB\(^{15}\). This suggests that major OligoS-F have a contact terminal
phosphate group, but minor OligoS-F may have a phosphoryl glycolate group at 3'-end. Therefore, the SB mainly involve C-O rather than P-O bond cleavage along the sugar-phosphate backbone of DNA.

Considering that the effective current density \((J_{\text{eff}})\) for SB decreases exponentially as a function of the electron penetration depth \((nh \text{ in nm})\)^{16}, we obtain:

\[
J_{\text{eff}}(n) = J_0 e^{-nh/AL}
\]  

(2)

where \(J_0\) is the incident current density of \((1.0 \pm 0.1) \times 10^{12} \text{ electrons cm}^{-2} \text{ s}^{-1}\), AL the attenuation length in nm, \(n\) the number of nucleotides in a given fragment and \(h\) the vertical rise per nucleotide of \(0.22 \pm 0.01 \text{ nm}\), based on the measurements of Ray et al^{17} and Lamont et al^{18}.

Owing to the linear dependence of the exposure-response, the yield of 5'-OligoS-F \((\gamma)\) can be estimated from the relation:

\[
\gamma(n) = \sigma_{\text{eff}} N_0 J_{\text{eff}}(h) t
\]  

(3)

where \(\sigma_{\text{eff}}\) is the average effective cross section for SB per nucleotide, \(N_0\) the initial number of OligoS within the exposure area of the electron beam \((N_0 = 0.55 \times 0.55 \times 3.14/4 \times 5.8 \times 10^{12} = 1.4 \times 10^{12} \text{ molecules})\) and \(t\) the exposure time of 600s.

Combining equations 2 and 3, we obtain:

\[
\gamma(n) = \sigma_{\text{eff}} N_0 t J_0 e^{-nh/AL}
\]  

(4)

Comparing equations 1 and 4, we retrieve the physical definition of the two fitting parameters:

\[
a = \sigma_{\text{eff}} N_0 t J_0
\]  

(5)
\[ b = \frac{h}{\text{AL}} \]  

Therefore, \( \sigma_{\text{eff}} \) and AL can be derived by fitting the yield of OligoS-F vs its length to equation 1 for each incident electron energy.

The inset of Fig. 3 shows that AL decreases exponentially with electron energy. The error bars represent the uncertainty range of the fitting parameter b. The uncertainty on the absolute values of the AL is estimated to be 10% from the absolute error on the quantification of the fragments from the gel. Thus considering errors from both the absolute and fitting uncertainties, the \( \text{AL} \) are listed in Table I. These values can be compared with those of Lamont and Wilkes, who used XPS to measure the AL of electrons of 50-1000 eV in SAM of \( n \)-alkanethiols (\( n \)=8-18) chemisorbed on gold. They found AL of 0.65 ± 0.12 nm at 50 eV and a minimum of 0.54 ± 0.04 nm at 100 eV. Although molecular structure of DNA is different from that of alkanes, the AL are similar in both substances. On the other hand, Monte Carlo simulations of the energy loss by electrons in solid DNA gives IMFP in \( \mu \text{g cm}^{-2} \) of 1.01 (7.5 nm), 0.23 (1.7 nm) and 0.13 (1 nm) for electrons of 25, 50 and 100 eV, respectively, which are much larger than our AL at 18 and 28 eV. The IMFP represents the average distance traveled by an electron of a given energy between inelastic collisions in a substance, while the \( \text{AL} \) is the average decay length of an effective electron current to cause a given event that decreases exponentially in a given substance. If elastic scattering effects were negligible, the IMFP and AL would be identical. Powell estimated that the AL could be smaller than the related IMFP by up to about 30%. Taking this percentage into consideration, we find that there is also good agreement between our values at 48 and 68 eV and those calculated at 50 and 100 eV for solid DNA. However, the IMFP at 25 eV derived from
Monte Carlo simulations is much longer than the AL at 18 and 28 eV measured in this study. This is not surprising since the simulations are based on optical constants and X-ray attenuation cross sections which are not related to LEE interactions. Furthermore in these calculations, the final energy for the calculation of the continuous-slowing down approximation range was taken to be 25 eV\(^1\). In general, owing to the lack of reliable cross sections at low energies, to be introduced as basic data into Monte Carlo codes, mathematical simulation of radiation damage often assume that electrons of energies below a certain threshold have negligible energy loss cross sections.

Figure 5 shows the dependence of \(\sigma_{\text{eff}}\) on the incident electron energy. Error bars only represent the uncertainty range of the fitting parameters \(a\). The absolute uncertainties in \(\sigma_{\text{eff}}\) are estimated to be 25% of the derived values. They arise from the absolute errors on (1) the incident current density (10 %), (2) target area (5 %), (3) recovery of fragments (5 %) and scintillation counting (5 %). The \(\sigma_{\text{eff}}\) are listed in Table I considering both absolute and fitting uncertainties.

The energy dependence of \(\sigma_{\text{eff}}\) shows a resonance peak of magnitude \((2.8 \pm 0.9) \times 10^{-17}\) cm\(^2\) at 18 eV superimposed on a monotonically rising curve. This behaviour has been seen in the yield function for SSB and DSB in plasmid DNA induced by LEE impact\(^4\). Maxima have been reported in these yields at 19 eV and 22 eV, respectively. A peak also appears near 23 eV in the yield function of LEE-stimulated \(H^+\) desorption from condensed-phase deoxyribose analogues\(^10\). This peak has been interpreted to arise principally from decay of a transient anion into an electronic excited state, which dissociates into \(H^-\) and the corresponding positive ion radical. Sugar radical has been suggested as the main source of DNA strand scission\(^20\). On the other hand, according to
a recent chemical analysis\textsuperscript{7} of the damage induced by 10 eV electron impact on thin solid films of oligonucleotide tetramers CGTA and GCAT, cleavage of phosphodiester bonds depends on the site and the nucleobase, thus appears to involve initial trapping of an electron by the nucleobase followed by the transfer of the electron to the phosphodiester bond. In their study oligonucleotide tetramers were physisorbed on tantalum and most likely lay flat on the substrate\textsuperscript{7}, while in this study 50 mer oligonucleotides were chemisorbed on gold and were oriented and "standing up". Furthermore the SB observed in this study does not show observable preference to individual nucleobase. Therefore the peak at 18 eV, shown in Fig. 5, may arise from a different resonance process, eg. direct electron attachment to the sugar-phosphate backbone, followed by dipolar dissociates into $H^+$ and the corresponding positive ion radical, leading to C-O bond cleavage\textsuperscript{10}.

Since the technique described in the article is the first from which it is possible to extract an average cross section per scattering site of a large biomolecule, it is difficult to compare our results with those of other experiments. The only experimental cross sections for LEE induced damage to DNA have been recorded by Boudaïffa et al\textsuperscript{3}. At 10, 30 and 50 eV, they obtained effective cross sections per plasmid for a five-layer thick film of plasmid DNA. When normalized per nucleotide these cross sections translate to $4.1 \times 10^{-19}$, $4.7 \times 10^{-19}$ and $5 \times 10^{-19}$ cm$^2$, respectively; i.e. they are one to two orders of magnitude larger than ours at 8, 28 and 48 eV, i.e. $(3 \pm 1) \times 10^{-18}$, $(2.0 \pm 0.7) \times 10^{-17}$ and $(3.2 \pm 1.1) \times 10^{-17}$ cm$^2$. This is expected since an effective cross section for a five-layer film of plasmids contains non-negligible contributions from energy loss electrons. Furthermore, variation of film thickness and clustering as well as the lower purity in the plasmid experiment should lower the cross sections. The single strandness and the
different topology of an oligonucleotide versus a supercoiled plasmid of DNA may also contribute to the differences. In any case, owing to the more rigid experimental conditions and the reduced sources of errors in our experiment, the present cross sections should be considered more reliable.

IV. Summary

We have developed a method to measure AL and effective cross sections per scattering site for LEE-induced damage in biomolecules. The method has been applied to the determination of such parameters for SB in SAM of single stranded oligonucleotides in the range of 8 - 68 eV. The effective cross sections and AL extracted from our experiment are not necessarily transportable to electron scattering from oligonucleotides found in different environments. However, as long as changes in local environment do not appreciably modify LEE interactions, we expect similar cross sections for any film of oligonucleotides having thicknesses greater than the AL shown in Fig. 3 and for oligonucleotides lying within other media. Comparison of our experimental AL at 28 eV, with those obtained at about the same energy from Monte Carlo calculations, clearly shows the need of suitable LEE cross sections to simulate radiobiological damage. The present data should therefore be useful to understand and predict DNA damage induced by radiation and simulate DNA damage in cells via Monte Carlo calculations.

ACKNOWLEDGMENTS

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II. RESULTS

Table I  Attenuation length and effective cross section for SB in SAM of oligonucleotides chemisorbed on gold as a function of electron energy

<table>
<thead>
<tr>
<th>Incident electron energy (eV)</th>
<th>Attenuation length (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Effective cross section for SB (× 10&lt;sup&gt;17&lt;/sup&gt; cm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.5 ± 0.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>18</td>
<td>1.6 ± 0.3</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>28</td>
<td>1.5 ± 0.3</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>38</td>
<td>1.2 ± 0.2</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>48</td>
<td>1.0 ± 0.2</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>58</td>
<td>1.0 ± 0.2</td>
<td>4.4 ± 1.4</td>
</tr>
<tr>
<td>68</td>
<td>0.8 ± 0.1</td>
<td>5.1 ± 1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> The errors represent the sum of the uncertainty range of the fitting parameter b and 10 % absolute error in gel quantification.

<sup>b</sup> The errors represent the sum of the uncertainty range of the fitting parameter a and 25 % absolute error in the measurements.
FIG. 1. Representation of a self-assembled monolayer (SAM) of a thiolated oligonucleotide chemisorbed on a gold substrate and its exposure to an electron beam.
FIG. 2. Dependence of total yield of LEE-induced 5'-oligonucleotide fragments on the amount of 58 eV electrons incident at a constant current density of $1.2 \times 10^{12}$ electrons cm$^{-2}$ s$^{-1}$. Error bars represent the absolute uncertainty of the measurements.
FIG. 3. Dependence of yield of LEE-induced 5'-oligonucleotide fragments on their length for electron energies of 8 (▲), 28 (○) and 68 eV (■). The curves represent fits to equation 1. The inset shows the dependence of the attenuation length (AL) on LEE energy. The error bars represent the uncertainty range of the fitting parameter b.
FIG. 4. Denaturing polyacrylamide gel of oligonucleotides. Lanes starting from the left show molecular size ladder generated from G+A chemical sequencing reactions of 5'- (GCTA)$_{12}$GC(CH$_2$)$_3$-SS-(CH$_2$)$_3$-OH-3' (GCTA)$_{12}$GC(CH$_2$)$_3$-SS-(CH$_2$)$_3$-OH-3', 5'-end oligonucleotide fragments formed by exposing 5'-$^{32}$P-(GCTA)$_{12}$GC(CH$_2$)$_3$-S-Au to low energy electrons of 0, 4, 8, 12, 18, 28, 38, 48, 58 and 68 eV, molecular size ladder generated from G+A chemical sequencing reactions of 5'-CATTAATGCTATGCGAGAAAAATCTTTAG, as well as 5'-(GCTA)$_{12}$GC(CH$_2$)$_3$-SS-(CH$_2$)$_3$-OH-3’ without sample manipulation.
FIG. 5. Energy dependence of the effective cross section for the induction of strand breaks by LEE in self-assembled monolayer of 3'-thiolated oligonucleotides. Error bars represent the uncertainty range of the fitting parameter $a$ (see text).
III. DISCUSSION

III.1. Is it possible to convert a quantum yield obtained by LEE beam experiments to a G value?

The direct conversion of the quantum yields of $\sim 7.5 \times 10^{-3}$ and $\sim 8 \times 10^{-4}$ SSB per incident electron at incident electron energies of 2.2 and 10 eV gives G values of 35 and 0.8 nmol J$^{-1}$, respectively (Martin et al. 2004; Boudaiffa et al. 2000b). These values are significantly smaller than the 86-247 nmol J$^{-1}$ of SSB for SE with average energy of 5.8 eV obtained in Article No. 3. This is not surprising given the problems related to charging and the energy imparted both to the DNA film and the metal substrate in LEE beam experiments. As shown in Fig. II-9, Al$\alpha$ X-ray induced SE-emission from tantalum produces a large amount of LEE. This alternative LEE source allows direct comparison of the yield of products induced by high-energy photons and LEE under identical experimental conditions. Exposure of metals to X-rays produces backscattering SE. SE is composed of LEE with the peak close to 1eV and fast SE, eg. Auger and photoelectrons (Henke et al. 1981). In order to have predominant LEE in SE and high quantum yields, it is better to use higher Z metal and lower energy X-rays eg. ultrasoft X-rays.

III.2. Why are low energy SE more efficient than X-ray photons for induction of DNA SSB and DSB?

Article No. 3. shows that SE with an average energy of 5.8 eV are more efficient than X-ray photons for inducing DNA SSB and DSB with an enhancement factor of at least 1.5 and 1.6, respectively. There are a number of mechanisms which can account for these.
differences, although the details can not be determined exactly at this stage. \( Al_\alpha \) X-rays interact with DNA mainly via the photoelectric effect and produce photoelectrons as well as Auger electrons. The absorption of electromagnetic radiation leads to the excitation (ca. 20\%) and ionization (ca. 80\%) of DNA (Sanche, 2002). The fast-electrons from ionization react essentially via the emission of virtual photons (Shimamura and Takayanagi, 1984) so that both the initial photon interaction and the fast secondary electrons produce ions and excited vibrational and electronic states within DNA in similar proportions. X-rays also produce LEE in the ionization process, but a large portion of the deposited energy flows into the creation of an ion or a hole, a mechanism which is practically absent in the case of LEE damage. More specifically, the studies of Razskazovskiy et al (2003) suggest that ionization of DNA, which takes a considerable amount of energy, gives rise to sugar centered radicals as precursors to SBS. Whereas X-rays and fast electrons produce electronic and vibrational excitation in similar proportion, the relative abundance of electronic excitations may be larger for LEE due to the formation of transient anions. Such a difference would result in a higher number of dissociative states produced per unit energy deposited by LEE. In any case, due to polarization and the formation of transient anions, it takes less energy to break a chemical bond with a LEE than with a photon. The threshold energy for breaking a DNA strand with a photon lies around 7 eV (Folkard et al. 2000), whereas a LEE induces SSB in DNA by DEA via the formation of shape resonances located at 0.8 and 2.3 eV (Martin et al. 2004). Core-excited resonances induce SSB and DSB (Boudaïf et al. 2000b) from 5 to 15 eV. These resonances consist of an electron temporarily captured by the positive electron affinity of an electronic excited state within DNA. The quantum yield of DNA
SSB at 0.8 eV is 15 fold that around 10 eV, suggesting that low-lying shape resonances in DNA are highly efficient for breaking the phosphodiester backbone. Thus, SE emitted from tantalum with an energy distribution peaking around 1.4 eV can cause considerable DNA SB via DEA.

III. 3. Are G values for the induction of DNA SSB and DSB for SE emission from Ta exposed to Al$_{ka}$ X-rays applicable to SE induced by X rays in a general biological environment?

The interaction of Al$_{ka}$ X-rays with DNA is mainly via the photoelectric effect, resulting in photoelectrons (~400-1455 eV, eg. Na 1s, 415 eV; O 1s, 954 eV; N 1s, 1086 eV; C 1s, 1202 eV; P 2s, 1296 eV; P 2 P3/2, 1356 eV; Na 2s, 1422 eV; Na, 2p, 1455 eV) and Auger electrons (Na, 22 and 987 eV; N, 397 eV; O, 506 eV) (Wagner et al. 2000). The most probable energy loss of primary electrons is 23 eV, resulting in the energy of SE most likely below 10 eV (LaVerne and Pimblott, 1996). Al$_{ka}$ X-ray-induced SE from Ta have an average energy of 5.8 eV. Thus, SE from Ta are similar to those from DNA. Therefore, the derived G values for DNA damage by SE are applicable to DNA in biological environments in the absence of a metal substrate.

III.4. Does ionizing radiation cause DNA interduplex CL in cells?

DNA is highly condensed in mitotic chromosomes. Figure III.1 shows the step-by-step packing of DNA from DNA into a chromosome (Alberts et al. 1998). The linear packing ratio is defined as the ratio between the length of extended DNA and the length of the structure that contains it and serves as a measure of DNA compaction (Sumner 2003). The packing ratios for nucleosome, chromatin fiber, interphase chromosome and
mitotic chromosome increase from about 6, to 40, 1000 and 10000, respectively (McClean, 1997). The local DNA concentration may be also used to describe the DNA compaction. Microscopy measurements showed that the local concentration of DNA in the interphase nucleus, in the metaphase chromosomes of human fibroblasts and in resting lymphocytes are 0.10, 0.16 and 0.12 g cm$^{-3}$, respectively (Daban, 2003). The above local concentrations of DNA refer to both the volume occupied by the DNA and the histone octamer core of nucleosome and other proteins. Excluding the volume contributed by histones, the DNA density is 1.7g cm$^{-3}$ (Freifelder, 1987), higher than the density of a dry DNA film (Lee et al. 1987). Thus the possibility that two DNA duplexes have close contacts should be high for interphase nucleus and chromosome, especially mitotic chromosomes. Furthermore, during DNA recombination and replication, chances for both close contacts of two DNA duplexes and partial unwinding of the DNA double helix are high. Therefore the potential exists for interduplex CL induction by ionizing radiation during DNA replication, recombination and mitosis. Multimeric concatemers were reported to be formed in vitro during recombination (Yang et al. 1999; Willer et al. 2000) and replication processes (Dronkert and Kanaar, 2001). It will be important to study whether and how X-rays might induce DNA interduplex CL in vivo during recombination, replication and chromosome condensation, as well as whether and how they are repaired.
Fig. III.1 Levels of Chromatin Packing (Alberts et al. 1998; Pollard et al. 2002)
III.5. Back-scattered SE from metal surfaces exposed to clinical X-rays enhance DNA SSB, DSB, and CL. What is the potential impact of this enhancement on the clinical practices of X-ray diagnosis and radiotherapy?

II. 4 shows that the enhancement factor (EF) of back-scattering SE on the induction of SSB, DSB and CL in a thin film (~30 nm or 4.7 μg cm$^{-2}$ thick) of DNA with two hydration layers ($\Gamma=21$), similar to those in cells, is about 10. The enhancement is due to both slow SE and fast SE. Slow SE account for 40 % of SE, but only carry ~5 % of the total SE energy, with a kinetic energy maximum around 1.4 eV. The fast SE (average energy of ~2 keV) account for ~60 % of SE but carry ~95 % of total SE energy. Most of the fast SE have penetration lengths >12.5 μg cm$^{-2}$ and should escape the thin film of DNA with more than 80 % of their original kinetic energy. The enhancement effect is expected to extend to 12 μm (about 1 cell diameter), considering the maximum SE energy of 30 keV (Pimblott and Siebbeles, 2002). The enhanced dose and damage to interface tissue caused by metal implants, gold tooth crowns and contrast media etc. probably should be considered for X-ray diagnosis and radiotherapy. Since the quantum yield of SE is scaled with photon energy and absorption coefficients of metals, the enhancement effect might be alleviated by adjusting photon energy or using low Z instead of high Z metal. On the other hand, the application of this enhancement (radiosensitization) effect to radiotherapy may be even more important. For example, gold nanoparticles were reported to enhance the efficiency of radiotherapy in mice (Hainfeld et al. 2004) and enhance DNA strand breaks (Foley et al. 2005).
III.6. The application of effective cross sections per nucleotide for DNA SB by LEE to radiation protection and radiotherapy.

Monte Carlo simulations are not only used in predicting low dose rate low dose radiation effects for radiation protection (Ballarini et al. 2002; Ballarini and Ottolenghi, 2003, 2004), but are also widely applied for radiotherapy treatment planning (Wigg, 2001; Lehmann et. al. 2005). These simulations often include DNA damage component to predict biological effects. However, owing to the lack of reliable cross sections for DNA damage and attenuation length at low energies, to be introduced as basic data into Monte Carlo codes, mathematical simulations of radiation damage often assume that electrons of energies below a certain threshold have negligible energy loss cross sections. Such an assumption results in significantly longer electron attenuation lengths at low energy (as seen in Article 5). We may predict that the assumption also causes errors in predicting DNA damage, especially those by LEE. Article 5 describes a method for measuring AL and effective cross sections per scattering site for LEE-induced damage in biomolecules. Such a technique is the first from which it is possible to extract an average cross section per scattering site of a large biomolecule. Therefore, our effective cross sections of DNA damage per nucleotide by LEE provide important input parameters for Monte Carlo simulations used in radiation protection and radiotherapy.
IV. CONCLUSION/PERSPECTIVE

This research project resulted in the development of three methods for the investigation of DNA damage induced by low energy secondary electrons and X-rays, and four major findings.

For the direct comparison of DNA damage induced by SE and X-ray photons in the UHV chamber and under identical experimental conditions, two methods were developed. First, radiochromatic films and color scanners were used to measure the absorbed dose of ultrasoft X-rays in dry DNA under UHV. Second, both monolayer and thick (20 μm) films of dry pGEM®-3Zf(-) plasmid DNA deposited on tantalum foil were exposed to Alkα X-rays under UHV. For monolayer DNA, the damage was induced mainly by low energy SE emitted from the tantalum. For the thick films, the damage was induced chiefly by X-ray photons. Since the energy spectrum of the SE has a peak at 1.4 eV and an average energy of 5.8 eV, Alkα X-ray induced SE emission from tantalum was used as a source of LEE.

Based on the above methods, it was found that SE are more efficient than X-ray photons in causing DNA SSB and DSB; i.e. the G values for SSB and DSB induction by SE are at least 1.5 and 1.6, and could be as high as 4.3 and 4.4 times, of those for X-ray photons for the same damage, respectively. It was also discovered that Alkα X-rays induce DNA inter-duplex CL in dry DNA, which were detected using restriction enzyme digestion as well as both neutral and alkaline agarose gel electrophoresis. The G value for total DNA inter-duplex CL under UHV is 16 ± 4 nmol J⁻¹, which is 0.25 and 2.8 fold that of the G values for DNA SSB and DSB, respectively.
V. ACKNOWLEDGEMENTS

The enhanced DNA damage by the back-scattering SE from tantalum exposed to clinical soft X-rays in air were also investigated. The enhancement factor for SSB, DSB and CL induced by SE vs X-rays were derived to be: 5.0 ± 0.8, 3.6 ± 1.0 and 9 ± 4 at Γ ≈6, and 8 ± 1, 9 ± 2 and 5.0 ± 1.4 at Γ ≈21, respectively. This study provides a molecular basis for understanding the enhanced biological effects at interfaces during X-ray diagnostic examination and radiotherapy.

To measure attenuation lengths and effective cross sections per scattering site for LEE-induced damage in biomolecules, a method was developed using SAM of a 5′-32P-labelled 3′-thiolated oligonucleotide chemisorbed on gold exposed to a LEE beam. This technique is the first from which it is possible to extract an average cross section per scattering site of a large biomolecule. It is found that for 50-mer single stranded oligonucleotides the AL decreases with LEE energies from 2.5 ± 0.6 nm at 8 eV to 0.8 ± 0.1 nm at 68 eV, whereas σ_eff for SSB increases from (3 ± 1) × 10^-18 to (5.1 ± 1.6) × 10^-17 cm^2 within the same energy range. The energy dependence of σ_eff shows a resonance peak of (2.8 ± 0.9)×10^-17 cm^2 at 18 eV superimposed on a monotonically rising curve. Dissociative electron attachment to the sugar phosphate backbone is proposed to account for this maximum. The AL and σ_eff should be useful to understand and predict DNA damage induced by radiation and to simulate DNA damage in cells via Monte Carlo calculations.

In the future several studies can be continued. The following subjects should be investigated: 1) the direct comparison of DNA base damage and base release induced by SE and ultrasoft X-ray photons under UHV; 2) the direct comparison of DNA damage induced by back-scattering SE and clinical soft X-ray photons in air. This requires
developing a method to record the energy spectrum of the back-scattering SE; 3) the measurement of the AL and $\sigma_{\text{eff}}$ for double stranded oligonucleotides; 4) the measurement of $\sigma_{\text{eff}}$ for other types of DNA damage, eg. nucleobase damage; 5) the effect of nucleobase sequences and radiosensitizers such as bromouracile on $\sigma_{\text{eff}}$. 6) The possibility of DNA interduplex CL induction by radiation in vivo.
ACKNOWLEDGEMENTS

This research project and this thesis were completed under the supervision of Prof. Darel Hunting and Prof. Léon Sanche. I would like to thank Prof. Hunting for giving me the chance to study radiobiology, for his advice, kindness, generosity and help. I would also like to thank Prof. Sanche for his wonderful magic ideas and his patience in teaching me the physics of low energy electrons. Pierre Cloutier has been consistently giving me solid technical support through this project. I would like to say “merci beaucoup, Pierre!”

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I would like to thank: my husband Xifeng Li for his strong support for my academic pursuits; my son Augusta Li for his endless curiosity and gentle kisses; and finally my daughter Angela Li for her sweet smiles and big hugs. Thanks Xifeng, Augusta and Angela. You make my life meaningful and my belief strong.
VI. REFERENCES


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VI. REFERENCES


VII. APPENDIX

VII.1. γ-radiolysis of aqueous solutions of DNA as a tool to test the purity of plasmid DNA

The G value for DNA SSB formation and the rate constant for the reaction of DNA and ·OH are used to evaluate the purity of DNA. If contaminants such as protein and Tris exist in DNA samples, the derived G value should be lower whereas the derived rate constant should be larger than the reported values. ·OH is very reactive and is the precursor of DNA SSB during irradiation of dilute DNA solution (i.e. where the direct effect is negligible). Any contaminant should scavenge ·OH and protect DNA from SSB, thus artificially increasing the apparent rate constant (Milligan 1999).
Figure VII.1 The dependence of the formation of circular DNA (SSB) on the \(\gamma\)-absorbed dose in aerated aqueous solution containing 80 ng \(\mu\)L\(^{-1}\) plasmid DNA and 10 mM phosphate buffer, pH 7.8

Fig. VII.1 shows the linear dose dependence of the induction of DNA SSB in aerated aqueous solutions containing 80ng \(\mu\)L\(^{-1}\) plasmid DNA and 10 mM phosphate buffer, pH 7.8. The \(G\) value for DNA SSB was derived to be 50 \(\pm\) 3 nmol J\(^{-1}\), which is in good agreement with 58 nmol J\(^{-1}\) obtained for radiolysis of neutral aerated aqueous solutions containing 1.2 mg mL\(^{-1}\) DNA and 10 mM NaClO\(_4\) (von Sonntag, 1987).
Figure VII.2 The dependence of the formation of circular DNA (SSB) on the concentration of NaHCO$_3$ for the $\gamma$-radiolysis of aerated aqueous solution containing 17 ng $\mu$L$^{-1}$ plasmid DNA, 10 mM phosphate buffer (pH 7.8) and NaHCO$_3$. The absorbed dose is 0.9 Gy. The inset shows the dependence of the induction of SSB on the absorbed dose in aerated aqueous solution containing 28 ng $\mu$L$^{-1}$ plasmid DNA, 0.6 M NaHCO$_3$ and 10 mM phosphate buffer, pH 7.8.

Fig. VII.2 shows the induction of DNA SSB in $\gamma$-irradiated (0.9 Gy) aerated aqueous solutions containing 17 ng $\mu$L$^{-1}$ (50 $\mu$M of nucleotide concentration) plasmid DNA and 10 mM phosphate buffer, pH 7.8 and various concentrations of NaHCO$_3$. NaHCO$_3$ starts to protect DNA from SSB at a concentration of $\sim$1mM. As the
concentration of NaHCO$_3$ increases, the induction of SSB decreases. In aerated aqueous solutions, DNA SSB is mainly due to the H abstraction from sugar by $^\cdot$OH (von Sonntag, 1987). Bicarbonate anion is a weak $^\cdot$OH scavenger ($k=8.5\times10^6$ mol$^{-1}$dm$^3$s$^{-1}$) (Cai et al. 2001) and competes with DNA for $^\cdot$OH to form HCO$_3^\cdot$. HCO$_3^\cdot$ selectively oxidizes guanine and does not cause DNA SSB (Shavirovich et al. 2001). The efficiency of transfer of damage from oxidized guanine to sugar is about 0.5% (Milligan et al. 1999). The inset of Figure III-2 shows the linear dose dependence of the induction of DNA SSB in aerated aqueous solutions of 28 ng $\mu$L$^{-1}$ (85 $\mu$M of nucleotide concentration) plasmid DNA containing 0.6 M NaHCO$_3$ and 10 mM phosphate buffer, pH 7.8. In this solution the $^\cdot$OH scavenging capacity is $3.9\times10^6$ s$^{-1}$. The G value for DNA SSB is calculated to be $0.57 \pm 0.05$ nmol J$^{-1}$. Milligan et al.(1999) reported a G value for SSB of 2 nmol J$^{-1}$ for $\gamma$-radiolysis of aerated aqueous solutions containing 25 ng $\mu$L$^{-1}$ plasmid DNA, 0.03 mM NaBr and 10 mM phosphate buffer, pH 7.0. In their system, the $^\cdot$OH scavenging capacity is $3.3\times10^5$ s$^{-1}$. Considering the difference in $^\cdot$OH scavenging capacity between their and our studies, two G values compare fairly well. Using competitive reaction kinetics of HCO$_3^\cdot$ and DNA with $^\cdot$OH, the rate constant of our plasmid DNA reacting with $^\cdot$OH is derived to be $(7 \pm 1) \times10^8$ mol$^{-1}$dm$^3$s$^{-1}$ in terms of nucleotide concentration. This value is in good agreement with the reported value, eg. $(5.7 \pm 0.9) \times10^8$ mol$^{-1}$dm$^3$s$^{-1}$ in 10 mM sodium phosphate buffer, pH 7.2 (Schussler et al. 2005).

Both G values of DNA SSB and the rate constant of DNA reacting with $^\cdot$OH obtained in this study suggest that the purity of our plasmid DNA is comparable with
those used in other studies. The absorbance ratio A260/A280 of our plasmid DNA is 1.87 ± 0.02, which also confirms its high purity.

VII.2. The stability of plasmid DNA in UHV

![Graph showing the effects of DNA film thickness, tantalum surface, and storage time on supercoiled DNA percentage.](image)

Fig. VII.3 The effects of the thickness of DNA films, tantalum surfaces and storage time of DNA in UHV on the stability of plasmid DNA

The effects of tantalum surfaces and storage time of DNA in UHV on DNA stability were examined to optimize experimental conditions. Figure III.3 shows that the condition of tantalum surface significantly influences the stability of thin film of DNA during lyophilization. Thus, tantalum foil (Goodfellow, German) with a smooth surface, rather than tantalum plate (Goodfellow, German) with a rough surface, was chosen for
experiments. The percentage of supercoiled DNA was found to decrease as a function of storage time in UHV. Therefore, DNA samples were kept in vacuum for only about 2 hours to reach the required vacuum level of $1 \times 10^{-8}$ torr and to minimize the loss of supercoiled DNA.

VII.3. The stability of plasmid DNA in air

![Graph showing the stability of supercoiled DNA in air over time and concentration](image)

Fig. VII.4 The effects of DNA freezing methods, DNA concentrations and storage time on the stability of plasmid DNA in air, at 4°C and relative humidity of 45%

The storage of a plasmid DNA film on tantalum in air at room temperature (~25°C) and a relative humidity of 45% overnight resulted in 100% conversion of supercoiled to circular DNA. Thus, the DNA film was kept at 4°C to be hydrated and to
be exposed to soft X-rays. The effects of DNA freezing methods, DNA concentrations and storage time on the stability of plasmid DNA in air, at 4°C and relative humidity of 45% were examined. Figure III.4 shows that pre-freezing tantalum at -130°C before depositing DNA solutions retains a higher percentage of supercoiled DNA than depositing DNA on tantalum at room temperature following freezing both DNA and tantalum at -130°C. This may suggest that the tantalum surface reacts with DNA solution at room temperature. Similarly, when the tantalum is cleaned using ultrasonic bath before depositing DNA solution, lower percentage of supercoiled DNA is obtained. When the tantalum substrate is frozen before depositing DNA, the concentrations of DNA solutions have little effects on the stability of DNA, and the percentage of supercoiled DNA decreases slightly as the storage time at 4°C increases. Therefore, prefreezing the tantalum substrate and storing the DNA film at 4°C overnight for hydration were chosen for experiments.

VII.4. Why is it better to label oligonucleotides with $^{32}$P before exposure to an electron beam?

To avoid putting radioactive materials in an UHV chamber, in our initial experiments to measure attenuation lengths and cross sections using SAM of oligonucleotides chemiosorbed on gold, LEE-fragmented oligonucleotides are post-labelled (i.e. following irradiation) at the 5'-end using $^{32}$P ATP. However, we always observed a peak around a 10 mer oligonucleotide on the length distribution of LEE-fragmented oligonucleotides. Therefore, we used oligonucleotide sizing markers (8-32 bases, Amersham Biosciences) to test the relative labelling efficiency and recovery ratio.
of oligonucleotides from a Sephadex G50 microcolumn as functions of the length and concentration of oligonucleotides. The oligonucleotide sizing markers are composed of oligomers of the sequence d(GACT)n and d(GACT)nGT. The concentration of each oligomer is 0.175 pmol μL⁻¹, except that of the 20 mer oligomer with a 3-fold higher concentration. Since each oligomer has a G at the 5’-end, the labelling efficiency is not influenced by the 5’-nucleotide (van Houten et al. 1998). Various amounts of the oligonucleotide sizing marker were mixed with 0.37 MBq of [³²P] ATP and 10 U of T4 Polynucleotide kinase (usb®) in 25 μL of 1X One-Phor-All buffer plus (usb®), and were incubated at 37 °C for 45 min, then at 75 °C for 10 min to inactivate the kinase. The ³²P labelled oligonucleotides were separated by electrophoresis either directly or after passing through a microcolumn containing 450 μL of Sephadex G-50. For electrophoresis, a denaturing 20 % polyacrylamide gel containing 7 M urea (20×25cm) was used and run at a constant power of 20 Watts for 60 min. A storage phosphor screen was exposed to the gel overnight at -20°C and subsequently scanned with a 633 nm laser using a STORM860 system in the phosphorescence mode (Molecular Dynamics). The gel was quantified using ImageQuant software (Molecular Dynamics).

VII.4.1. The effect of oligonucleotide lengths on their recovery from a Sephadex G50 column

Figure VII.5 shows how the length of oligonucleotides and total concentrations of oligonucleotides affect their recovery ratios from a Sephadex G50 column. Generally the recovery ratio increases as the length and concentration of oligonucleotides increase. However, it appears that certain unknown factors also influence the recovery and result in low reproducibility.

168
VII.4.2. The effects of the length of oligonucleotides on the efficiency of 5'-end $^{32}$P labeling using T4 polynucleotide kinase

Figure VII.6 shows the effects of the length of oligonucleotides on the relative efficiency of 5'-end $^{32}$P labeling using T4 polynucleotide kinase. 13 labelling reactions were performed. The concentrations of $^{32}$P-ATP and T4 polynucleotide kinase as well as the reaction time were the same for all reactions. The ratio of $^{32}$P-ATP vs total oligomer concentration ranged from 2 to 45. The average relative labeling efficiency appears the same for oligomers containing 14 to 32 nucleotides, while the efficiency increases approximately linearly with the number (n) of nucleotides that an oligomer has, for n=8 to 12. The standard deviation of 13 values of labeling efficiency is very large. However,
it is not due to the difference in oligomer concentration used in each reaction. For example, in two duplicate reactions using 10 nM of oligomers, the relative labeling efficiencies show differences as large as the standard deviation of the 13 reactions. At this moment it is not certain whether locally non-homogeneous distribution of oligonucleotides, $^{32}$P-ATP and T4 polynucleotide kinase in the reaction solution causes the low reproducibility of each labeling reaction.

![Graph showing relative $^{32}$P-labeling efficiency vs length of oligonucleotides counting from the 5'-end (mer).](image)

**Figure VII.6** Effects of the length of oligonucleotides on the relative efficiency of 5'-end $^{32}$P labeling using T4 polynucleotide kinase. The open circles and error bars represent the average labeling efficiency and the standard deviation of 13 labeling reactions with the ratio of $^{32}$P-ATP vs total concentration of oligonucleotides ranging from 2 to 45. Both solid and open triangles represent reactions using 10nM of oligonucleotides.