Université de Sherbrooke

SENSITIZATION OF PLASMID DNA TO IONIZING RADIATION BY PLATINUM CHEMOTHERAPEUTIC DRUGS

by

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Thesis submitted to the Faculty of Medicine and Health Sciences for the degree of Doctorate of Philosophy (PhD) in Radiation Sciences and Biomedical Imaging
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ABSTRACT
Sensitization of Plasmid DNA to Ionizing Radiation by Platinum Chemotherapeutic Drugs
by Mohammad Rezaee
Department of Nuclear Medicine and Radiobiology

Thesis submitted to the Faculty of Medicine and Health Sciences for the degree of
Doctorate of Philosophy (PhD) in Radiation Sciences and Biomedical Imaging, Faculty of
Medicine and Health Sciences, University of Sherbrooke, Sherbrooke, Québec, Canada

Concomitant chemoradiation therapy based on platinum chemotherapeutic drugs (Ptdrugs) is a common treatment modality for several types of cancers and has dramatically
improved patient survival. The radiosensitization capacity of Pt-drugs results essentially from
their binding to nuclear DNA. Although several mechanisms such as increase in the radiation
damage to DNA and inhibition of their repair have been proposed, the contribution and
efficiency of the underlying molecular mechanisms of the radiosensitization remain unknown.
This PhD thesis determines the relative efficiency of Pt-drugs, in terms of the type of drug and
the quantity of Pt-DNA adducts, in the sensitization of DNA to the direct and indirect effects
of ionizing radiations, and elucidates the major mechanism responsible for this
radiosensitization. In particular, it addresses the role of low-energy electrons (LEEs),
hydroxyl radicals and hydrated electrons in the radiosensitization of DNA modified by Pt-
drugs. This thesis includes a review of the literature on the molecular basis of radiotherapy,
Pt-based chemotherapy, and their combination in cancer treatment. Five articles, on which I
am first author, are presented, and followed by a comprehensive discussion that integrates all
results and their implications in the clinic and future research.

With respect to the direct effect of radiation, LEEs are found to be the main species
responsible for the enhancement in DNA damage, particularly cluster damage including DSB
and interduplex cross-links. Irradiation of a 3199-bp plasmid DNA modified by an average of
2 Pt-drug adducts with 10-eV electrons results in significant increases in DSB formation by
factors of 3.1, 2.5 and 2.4, respectively, for carboplatin, cisplatin and oxaliplatin relative to
unmodified DNA. Irradiation of these samples with subexcitation-energy electrons (i.e., 0.5
eV) generates substantial number of DSB in the modified DNA, while no DSB is observed in
the unmodified DNA. Since 0.5 eV is well below that energy required for the electronic
excitation of organic molecules, dissociative electron attachment must be the main
mechanism responsible for the formation of strand breaks in the presence of Pt-adducts. For
indirect effects of radiation, our results show that both hydroxyl radicals and hydrated
electrons are responsible for the enhanced formation of damage in modified DNA. In the
presence of Pt-adducts, hydroxyl radicals mainly contribute to the SSB formation, while
hydrated electrons are the main species responsible for the DSB formation.

Our results indicate that carboplatin and oxaliplatin have higher efficiency than cisplatin
in the enhancement of radiation damage to DNA. At low frequencies of Pt-DNA adducts (i.e.,
less than 3.1x10^-4 adducts per nucleotide), radiosensitization of DNA, in terms of the damage
per adduct, increases by an order of magnitude compared with that at large frequencies of
adducts. In conclusion, Pt-drug sensitization is an extremely efficient means of enhancing the
formation of DNA DSBs by both LEEs and hydrated electrons created by ionizing radiation.

Key-words: Cisplatin, Carboplatin, Oxaliplatin, Electron, DNA damage, Radiosensitization
RÉSUMÉ

Sensibilisation de l'ADN plasmidique aux rayonnements ionisants par les médicaments chimiothérapeutiques platines

Par Mohammad Rezaee
Département de médecine nucléaire et de radiobiologie

Thèse présenté à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de philosophiae doctor (Ph.D.) en science des radiations et imagerie biomédicale, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

La radiochimiothérapie concomitante, basée sur les médicaments antinéoplasiques platines (Pt-antineoplasiques), est une modalité de traitement utilisé contre plusieurs types de cancers et a considérablement amélioré la survie des patients. Parmi ces médicaments anticancéreux, les analogues de platine sont les plus couramment utilisés. Leur capacité à radiosensibiliser résulte essentiellement de leur liaison à l'ADN nucléaire. Bien que plusieurs mécanismes aient été proposés tels que l'augmentation des dommages induits à l'ADN et l'inhibition de leur réparation, la contribution et l'efficacité des mécanismes moléculaires sous-jacents à la radiosensibilisation restent inconnus. La présente étude examine l'efficacité Pt-antineoplasiques à sensibiliser l'ADN aux rayonnements ionisants et détermine le rôle des électrons secondaires, des radicaux d'hydroxyles et des électrons hydratés dans ce processus. Cette thèse comprend un revue de des données scientifiques concernant la base moléculaire de la radiothérapie, de la chimiothérapie Pt-antineoplasiques et de leur combinaison dans le traitement de cancer. Cinq articles, dont je suis premier auteur, sont présentés suivis d'une discussion qui intègre mes résultats et leurs implications dans la clinique et la recherche future.

En ce qui concerne l'effet direct des radiations, les électrons de faible énergie s'avèrent être la principale espèce responsable de l'augmentation des dommages à l'ADN, en particulier les dommages multiples localisés, les CDBs et les pontages inter-brin. L'irradiation de plasmides de 3199 paires de bases, contenant en moyenne deux adduits Pt-ADN, avec des électrons de 10 eV conduit à une augmentation significative des CDBs par des facteurs de 3,1, 2,5 et 2,4, respectivement, pour le carboplatine, le cisplatine et l'oxaliplatine par rapport à l'irradiation des plasmides non modifiés. L'irradiation avec des électrons de 0,5 eV génère un nombre substantiel de CDBs dans les plasmides modifiés, alors qu'aucune CDB n'est observée dans les plasmides non modifiés. Puisque 0,5 eV est une énergie bien inférieure à celle nécessaire à l'excitation électronique des molécules organiques, l'attachement dissociatif de l'électron doit être le principal mécanisme responsable de la formation de cassures en présence de Pt-antineoplasiques. Pour les effets indirects des rayonnements, nos résultats montrent que les radicaux hydroxyles et les électrons hydratés sont, tous les deux, responsables de la formation accrue des dommages dans l'ADN modifié. En présence d'adduits Pt-ADN, les radicaux hydroxyles contribuent principalement à la formation de cassures simple brin, tandis que les électrons hydratés sont les principales espèces responsables de la formation de CDBs.

Nos résultats indiquent que le carboplatine et l'oxaliplatine sont plus efficaces que le cisplatine pour augmenter les dommages à l'ADN. À faible concentration de Pt-ADN (soit moins de 3,1x10^4 adduit par nucléotide), la radiosensibilisation de l'ADN, en termes de dommages par adduit, est d'un ordre de grandeur supérieure à celle aux concentrations élevées. En conclusion, l'ajout de Pt-antineoplasiques est un moyen extrêmement efficace d'augmenter la formation de CDBs dans l'ADN par l'intermédiaire des électrons de faible énergie et des électrons hydratés produits par les rayonnements ionisants.

Mots-clés: Cisplatine, Carboplatine, Oxaliplatine, Electron, dommages à l'ADN, Radiosensibilisation
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**Figure 1** Exposure-response curve for the formation of nicked circular (left) and linear (right) DNA configurations corresponding to the SSB and DSB, respectively, by 0.5-eV electrons in DNA unmodified or modified by cisplatin, carboplatin and oxaliplatin. The sample films were deposited on DNA. Data are means ± SD (standard deviation) from five measurements. They have been fitted by a least squares regression analysis.

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**Figure 3** (a) A double helix DNA (I) and the formation of a TNI on the phosphate group in the absence (II) and presence (III) of Pt-drugs. Formation of Pt-adducts such as interstrand crosslinks between two guanines, as shown here, causes to distort DNA conformation by unwinding and bending its double helix. Such distortion modifies physical and chemical stability of the DNA leading to the weakening of the chemical bonds, which could enhance the formation of a TNI and its decay into DEA. X corresponds to NH$_3$, NH$_3$ and C$_6$H$_{10}$(NH$_2$)$_2$ for cisplatin, carboplatin and oxaliplatin, respectively. dR denotes the deoxyribose moiety. (b) Schematic diagram showing potential energy curves for negatively-charged singly (curve I) and doubly charged phosphate groups (i.e., formation of a TNI) in the absence and presence of Pt-drugs (curves II and III, respectively). $R_E$ is the equilibrium internuclear
distances of the singly charged phosphate group, \( R_C \) and \( R_{C'} \) are the crossing points in unmodified and modified DNA, respectively. The presence of Pt-adducts is expected to shift the crossing point towards \( R_e \) (i.e., \( R_{C'} < R_C \)) resulting in enhanced DEA and subsequently formation of DNA strand breaks by subexcitation-energy electrons. F-C region and AD denote transition in Frank-Condon region and autodetachment, respectively.

Figure S1 Schematic view of the apparatus used to irradiate DNA samples with 1.486 keV Al K\(_\alpha\) X-ray photons. The apparatus comprises a chamber evacuated to pressure below 5 mTorr, connected to a pressure gauge (A) and an adjustable leak valve (B) connected to a nitrogen gas source. This valve stabilizes the nitrogen pressure at about 20 mTorr in the main chamber to control the plasma current. A negative potential of 3.4 kV is applied to a concave aluminum cathode (C) through a high-voltage electrical feedthrough (D) fixed in a glass-ceramic (Macor) support (E) and placed as a cap on a long quartz tube (F). A nitrogen plasma discharge with 5.5 mA current is formed between the cathode and an aluminum foil target (G). Aluminum atoms are ionized by electrons incident on the thin foil and characteristic K\(_\alpha\) X-rays with energy 1.486 keV are emitted outside the chamber through a flight tube (H) continuously flushed with helium gas at atmospheric pressure. X-ray traverse the helium gas and then a thin foil of Mylar (I) to enter a small chamber, where the plasmid DNA films deposited on the different substrates have been inserted on six aluminum plates of 44.5 mm diameter (J). These plates are fixed at different positions around a brass rotating disc (K) to allow irradiation of samples directly by X-rays, for different periods of time (i.e., various radiation doses) in the presence of specific amounts of gases or vapours introduced by valves (L). In the present experiments, the distance of 1.7 ± 0.05 mm between the Mylar foil and the surface of the plates is occupied by dry \( \text{N}_2 \) at atmospheric pressure. Lyophilized samples of plasmid DNA are placed very close to the Mylar foil to avoid too much photon absorption by the surrounding atmosphere. Furthermore, GAFCHROMIC® HD-810 radiochromatic dosimetry film (Advanced Materials Group of International Specialty Products Technologies Inc., Wayne, NJ, USA) were used to measure the incident photon fluence for each irradiation period.

Chapter II: Fifth Article

Figure 1 Dose-response curves for the formation of circular and linear DNA by \(^{60}\text{Co}\) γ-rays for the DNA and cisplatin DNA samples in 5 mM tris. Panels A and B indicate the curves for the circular DNA in
the samples saturated with N₂ and N₂O, respectively. The curves for the linear DNA are shown in the panels C and D for the samples saturated with N₂ and N₂O, respectively. Data in A – D are means ± SD from three experiments. They have been fitted by employing a least squares regression analysis.

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**Chapter III**

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**Figure III.3** Dose-response curves for the lose of circular (a) and the formation of linear DNA (b) by ⁶⁰Co γ-rays in the presence of cisplatin interstrand (square) and intrastrand (circle) CLs.
LIST OF ABBREVIATIONS

5-FU 5-flourouracil
A  Adenine
AD  Autodetachment
AE radionuclide Auger-electron emitting radionuclide
AEA  Adiabatic Electron Affinity
AL  Attenuation Length
amu  Atomic mass unit
bp  Base pair
C4'  Carbon atom at position 4 of deoxyribose molecule
cal  calorie
Carboplatin  cis-diammine(1,1-cyclobutane-dicarboxylato)platinum II
C-C  Carbon-Carbon
CCRT  Concomitant Chemoradiation Therapy
CHO  Chinese Hamster Ovary
Cisplatin  cis-diamminedichloroplatinum II
CL  Cross-link
C-N  Carbon-Nitrogen
C-O  Carbon-Oxygen
CRT  Chemoradiation Therapy
CS  Cross Section
DACH  Diaminocyclohexane
ddH2O  Distilled deionized water
DEA  Dissociative Electron Attachment
DMSO  Dimethyl Sulfoxide
DNA  Deoxyribonucleic Acid
DNA-PKcs  DNA-dependent protein kinase, catalytic subunit
DSB  Double-Strand Break
E. coli  Escherichia coli
EDTA  Ethylenediaminetetraacetic acid
EEL  Electron Energy Loss
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF</td>
<td>Enhancement Factor</td>
</tr>
<tr>
<td>ESD</td>
<td>Electron Stimulated Desorbed</td>
</tr>
<tr>
<td>eV</td>
<td>Electron-volt</td>
</tr>
<tr>
<td>F-C region</td>
<td>Frank-Condon region</td>
</tr>
<tr>
<td>FHX</td>
<td>5-flourouracil, hydroxyurea and radiation</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>G2-M phase</td>
<td>Gap2-Mitosis phase</td>
</tr>
<tr>
<td>HNC</td>
<td>Head and neck cancer</td>
</tr>
<tr>
<td>ICL</td>
<td>Interstrand cross-link</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma – Mass Spectroscopy</td>
</tr>
<tr>
<td>LEE (LEEs)</td>
<td>Low-Energy Electrons</td>
</tr>
<tr>
<td>LEET</td>
<td>Low-Energy Electron Transmission</td>
</tr>
<tr>
<td>LEPET</td>
<td>Low-Energy Photoelectron Transmission</td>
</tr>
<tr>
<td>LET</td>
<td>Linear Energy Transfer</td>
</tr>
<tr>
<td>MFP</td>
<td>Mean Free Path</td>
</tr>
<tr>
<td>ML</td>
<td>Monolayer</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MSA</td>
<td>Molecular Self-Assembly</td>
</tr>
<tr>
<td>N</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>NbP</td>
<td>Number of base pair</td>
</tr>
<tr>
<td>N7</td>
<td>Nitrogen at position 7 of purin bases</td>
</tr>
<tr>
<td>NA</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-Homologous End Joining</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Trans-R,R-1,2-diaminocyclohexane oxalate platinum II</td>
</tr>
<tr>
<td>p</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PLDR</td>
<td>Potentially Llthal damage repair</td>
</tr>
<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>Pt-DNA adduct</td>
<td>DNA adduct formed by Platinum-based chemotherapeutic drugs</td>
</tr>
<tr>
<td>Pt-drugs</td>
<td>Platinum-based chemotherapeutic drugs</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PtTC</td>
<td>Chloroterpyridine platinum</td>
</tr>
<tr>
<td>P-value</td>
<td>Probability value</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>S Phase</td>
<td>Synthesis Phase</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembly Monolayer</td>
</tr>
<tr>
<td>SB</td>
<td>Strand Break</td>
</tr>
<tr>
<td>SC</td>
<td>Supercoiled</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>SLDR</td>
<td>Sunlethal damage repair</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>Ta</td>
<td>Tantalum</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetic acid-EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TNI</td>
<td>Transient negative ion</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UHV</td>
<td>Ultrahigh vacuum</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VBE</td>
<td>Vertical binding energy</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
**LIST OF SYMBOLS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A</td>
<td>area</td>
</tr>
<tr>
<td>ε</td>
<td>Exposure</td>
</tr>
<tr>
<td>ϕ</td>
<td>Fluence</td>
</tr>
<tr>
<td>Y</td>
<td>Yield of damage per absorbed dose</td>
</tr>
<tr>
<td>Y'</td>
<td>Yield of damage per incident electron</td>
</tr>
<tr>
<td>$(dT/\rho dx)_c$</td>
<td>Collision stopping power</td>
</tr>
<tr>
<td>$\sigma_c$</td>
<td>Cross section of electron capture</td>
</tr>
<tr>
<td>$\sigma_{DEA}$</td>
<td>Cross section of dissociative electron attachment</td>
</tr>
<tr>
<td>$P_S$</td>
<td>Survival probability of a TNI</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

I.1. Concomitant Chemoradiation Therapy:

Concomitant chemoradiation therapy (CCRT) is the concurrent combination of chemotherapeutic drugs with ionizing radiation. CCRT is now applied to cancer patients as a primary treatment modality. In addition, this is a standard method for adjuvant and neoadjuvant therapy, when surgery is the primary treatment (Seiwert, Salama and Vokes, 2007). Table I.1 shows a brief overview of malignancies that are frequently treated by CCRT. This treatment modality has been reported to increase the killing of tumour cells, to improve the locoregional control of tumours, to preserve the organ affected by tumour cells, and to enhance patients’ survival (Boscolo-Rizzo, Gava, Marchiori, Baggio and da Mosto, 2011; Candelaria, Garcia-Arias, Cetina and Dueñas-Gonzalez, 2006; Peters III et al., 2000; Salama, Seiwert et Vokes, 2007; Samant et al., 1999). The most significant clinical rationale supporting the administration of CCRT is the role of chemotherapeutic drugs as radiosensitizers that enhance local therapy while also providing systemic therapy (Seiwert, Salama and Vokes, 2007).

The interaction of radiation with anticancer drugs occurs at molecular, cellular and tissue levels via various proposed mechanisms (Hennequin and Favaudon, 2002; Spalding and Lawrence, 2006). At the molecular level, CCRT can result in an increase or modification in the radiation-induced damage to DNA. Concomitant exposure of cultured cells to radiation and etoposide (i.e., a Topoisomerase II inhibitor), for example, has been shown to enhance the formation of double-strand break (DSB) due to the conformation changes in chromatin and DNA (Foray, Arlett and Malaise, 1997; Yu, Giocanti, Averbeck, Megnin-Chanet and Favaudon, 2000). Chemotherapeutic agents can also inhibit or alter DNA repair processes leading to the conversion of sublethal DNA damage induced by radiation to lethal damage. Antimetabolite drugs such as 5-fluourouracil and gemcitabine, for instance, radiosensitize tumour cell by inhibition of DNA synthesis and repair through depletion of nucleotide triphosphate pool and thymidylate synthase inhibition (Lawrence, Tepper and Blackstock, 1997; McGinn and Lawrence, 2001).
Table 1.1. Overview of cancers and indications of drugs used in different concurrent chemoradiotherapy. (Reprint with permission from Macmillan Publishers Ltd: Nature Clinical Practice Oncology (Seiwert, Salama and Vokes, 2007), Copyright 2007).

<table>
<thead>
<tr>
<th>Disease entity</th>
<th>Indication and treatment</th>
<th>Commonly used agents</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper aerodigestive tract cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck cancer</td>
<td>Locally advanced HNC—primary or adjuvant treatment</td>
<td>Cisplatin, 5-FU, FAH, cetuximab</td>
<td>Improved organ preservation and survival compared with radiation alone</td>
</tr>
<tr>
<td>Non-small-cell lung cancer</td>
<td>Stage III, nonoperable nonmetastatic disease</td>
<td>Cisplatin, carboplatin/paclitaxel, cisplatin/etoposide</td>
<td>Curative approach in poor surgical candidates or IIIB disease</td>
</tr>
<tr>
<td>Small-cell lung cancer</td>
<td>Limited stage disease</td>
<td>Cisplatin/etoposide</td>
<td>Curative in ~20% of patients</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>Locally advanced disease</td>
<td>Cisplatin/5-FU</td>
<td>Survival benefit, increased cure rates, organ preservation</td>
</tr>
<tr>
<td>Gastrointestinal malignancies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal cancer</td>
<td>Neoadjuvant</td>
<td>5-FU</td>
<td>Improved sphincter preservation, decrease in local and distal failures</td>
</tr>
<tr>
<td>Anal cancer</td>
<td>Mainstay of curative treatment</td>
<td>5-FU, MMC</td>
<td>Improved organ preservation</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Adjuvant</td>
<td>Cisplatin, 5-FU</td>
<td>Some data indicate a survival benefit</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Adjuvant, unresectable locoregionally advanced tumors</td>
<td>5-FU</td>
<td>Improved locoregional control, possibly a survival benefit</td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>Adjuvant, unresectable locoregionally advanced tumors</td>
<td>5-FU</td>
<td>Some data indicate a survival benefit</td>
</tr>
<tr>
<td>Gynecological and genitourinary cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>Primary modality</td>
<td>Cisplatin, 5-FU, hydroxyurea</td>
<td>Improved local and distal control, organ preservation</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>Primary modality</td>
<td>Cisplatin</td>
<td>Improved local control</td>
</tr>
<tr>
<td>Other cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Adjuvant</td>
<td>Temozolomide</td>
<td>Survival benefit</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>Neoadjuvant</td>
<td>Doxorubicin</td>
<td>Downstaging, improved organ preservation</td>
</tr>
</tbody>
</table>

At the cellular level, CCRT can interfere with cell cycle and promote apoptosis. The cooperative effects of chemotherapy and radiotherapy on cytokinesis have been reported to increase cellular toxicity leading to cell cycle arrest and apoptosis (Hennequin and Favaudon, 2002). The anticancer drugs inducing DNA damage in the synthesis (S) phase of the cell cycle such as Topoisomerase I inhibitors (e.g., camptothecin) has been shown to sensitize cells in S phase to ionizing radiation resulting in cell death (Hennequin, Giocanti, Balosso and Favaudon, 1994). Some anticancer drugs such as paclitaxel and docetaxel are able to block the cell cycle at the G2-M phase, leading to synchronization of the cell cycle at the most radiosensitive phase, and increase the efficacy of the subsequent radiotherapy (Hei, Piao, Geard and Hall, 1994).

At the tissue level, radiation can improve tumour retention of chemotherapeutic drugs and can increase vascular permeability, resulting in an increase in the concentration of chemotherapeutic drugs in the tumour tissue (Spalding and Lawrence, 2006). In
addition, reduction of tumour volume due to treatment with one modality can result in reoxygenetation and thus enhancement of the tumour cell sensitivity to radiotherapy or chemotherapy (Mason et al., 1999; Milas, Hunter, Milross, Saito and Peters, 1995).

Over the last thirty years, many studies have been devoted to finding an optimum protocol for a synergistic combination between chemotherapy and radiotherapy (i.e., timing, choice and dosage of the drugs and radiation) by attempting to understand the basic mechanisms responsible for the combination, however these mechanisms and their contribution to the synergistic action still remain the subject of active investigation.

1.2. Platinum Chemotherapeutic Drugs:

Cisplatin, carboplatin and oxaliplatin (Fig. 1.1) are platinum-based chemotherapeutic drugs (Pt-drugs) that are widely used in cancer treatment. Cisplatin (cis-diamminedichloroplatinum II) has significant activity against several forms of cancer, including ovarian, cervical, esophagus, non-small-cell lung, bladder, head and neck cancer (Boulikas, Pantos, Bellis and Christofis, 2007). However, severe side effects including nephrotoxicity, neurotoxicity, emetogenesis and ototoxicity as well as tumour resistance to the drug limit its clinical applications (Kelland, 2007). The latter mainly arises from reduced uptake, increased efflux, inactivation by sulphur-containing molecules such as glutathione and metallothionein, increased capability to repair Pt-drugs-DNA adducts (Pt-DNA adducts), and tolerance to these adducts (Wang and Lippard, 2005).

Carboplatin (cis-diammine (1,1-cyclobutane-dicarboxylato) platinum II), a second generation of the Pt-drug, is less toxic to kidney, gastrointestinal tract and nervous system compared to cisplatin (Boulikas, Pantos, Bellis and Christofis, 2007). The spectra of cancers that can be treated by carboplatin are similar to those of cisplatin and it has thus often replaced cisplatin (Go and Adjei, 1999). The third generation of Pt-drugs, oxaliplatin (trans-R,R-1,2-diaminocyclohexane oxalate platinum II), has a different pattern of sensitivity and activity against cisplatin-resistant cancers (Boulikas, Pantos, Bellis and Christofis, 2007). Such distinctive characteristics are believed to result from different recognition and repair processes for oxaliplatin-DNA adducts compared to
cisplatin and carboplatin (Di Francesco, Ruggiero and Riccardi, 2002). Oxaliplatin has lower nephrotoxicity and ototoxicity than cisplatin; however neurotoxicity is its most severe adverse effect (Lersch et al., 2002). It is a standard chemotherapeutic drug for the treatment of colorectal cancer, and is usually combined with other chemotherapeutic drugs such as 5-fluorouracil and leucovorin (De Vita et al., 2005).

Pt-drug molecules enter cells mainly via passive diffusion, but their uptake is slower than other small chemotherapeutic molecules due to their high polarity (Gately and Howell, 1993). They are also transported actively into cells through both copper transporter-1 and organic cation transporters (Kelland, 2007). Inside the cell, a Pt-drug molecule converts to chemically reactive forms via its hydrolysis, in which one or two ligands are substituted by water molecules (Ciarimboli et al., 2005; Wang and Lippard, 2005). The activated Pt-drug reacts with cellular components through ligand exchange at the platinum atom (Hannon, 2007; Schwietert and McCue, 1999). Owing to the slow kinetics of the hydrolysis reaction (e.g., $t_{1/2} \approx 4$ h for cisplatin), the drug can diffuse through the cytoplasm, enter the nucleus, and react with DNA (Jung and Lippard, 2007). Carboplatin has a less labile leaving group (i.e., the cyclobutane di-carboxylate ligand) than the chloride and oxalate ligands in cisplatin and oxaliplatin, respectively; hence, it shows a lower reactivity to hydrolysis and to reaction with other biomolecules, thus permitting the administration of larger doses and subsequently a greater accumulation inside the cell nucleus relative to both cisplatin and oxaliplatin (Go and Adjei, 1999). Owing to the lower reactivity of carboplatin, on the other hand, higher concentrations are required to achieve the same cytotoxic effects as for cisplatin (Kelland, 2007).
Fig. 1.2. Schematic for the formation of various cisplatin-DNA adducts, including intrastrand cross-links, interstrand cross-link, DNA-Protein cross-link and mono-functional binding to guanine. Carboplatin and oxaliplatin also form the same type of DNA adducts as cisplatin.

The primary target of Pt-drugs is nuclear DNA, although other cellular components such as RNA, proteins and membrane phospholipids interact with the drugs (Jung and Lippard, 2007; Reedijk, 2009). Pt-drugs specifically bind to the nitrogen atom at position seven (N7) of the imidazole ring of the purine bases (i.e., the most accessible moiety with nucleophilic characteristics in a double-stranded DNA), mainly guanine (G) and lesser amount to Adenine (A). There are two main reasons for the preferential binding of Pt-drugs to G: (1) Under physiological conditions, N7 of G has higher nucleophilic tendency than that of A since it is more basic than A, and (2) the reaction rate for Pt-drug binding at N7 of 6-oxopurines (e.g., G) increases, by a factor of 1.6, compared to N7 of 6-aminopurines such as A, owing to the sterically hinderance of the respective amine group (Martin, 1999). Binding of Pt-drugs to DNA forms various DNA
adducts including intrastrand cross-links (CLs) [1,2-d(GpG), 1,2-d(ApG), and 1,3-
d(GpNpG)], interstrand CL, and monofunctional binding to guanine (Fig. 1.2) (Jamieson and Lippard, 1999; Kelland, 2007). Despite the similarity in the types of DNA adducts, each Pt-drug produces different proportions of the specific adducts. Cisplatin and oxaliplatin, for instance, mostly form 1,2-d(GpG) intrastrand CL, whereas the major carboplatin adduct is 1,3-d(GpNpG) intrastrand CL (Todd and Lippard, 2009). The adducts distort the conformation of DNA by unwinding and bending, causing the destabilization of the double helix (Jung and Lippard, 2007; Kelland, 2007; Poklar, Pilch, Lippard, Redding, Dunham and Breslauer, 1996). Structural analysis of these Pt-DNA adducts by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy has shown that each adduct distorts DNA structure in a distinctive manner. For example, intrastrand CLs bend DNA duplex toward the major groove resulting in a wider and shallower minor groove (Jung and Lippard, 2007), whereas interstrand CL bends DNA toward the minor groove leading to a widening of the major groove (Coste et al., 1999).

The presence of DACH ligand in oxaliplatin molecule also results in several conformational differences between DNA adducts formed by oxaliplatin and cisplatin, predominantly due to the interaction between DACH ligand and DNA constituents (Sharma, Gong, Temple, Bhattacharyya, Dokholyan and Chaney, 2007).

Such distorted DNA is recognized by several cellular proteins that activate various signal transduction pathways, some of which lead to DNA-damage recognition and repair (e.g., high-mobility group proteins) but others mediate the cytotoxicity of Pt-drugs resulting in cell cycle arrest and apoptosis (Jung and Lippard, 2007). Nucleotide excision repair (NER) is the major pathway known to remove Pt-drugs from DNA (Ferry, Hamilton and Johnson, 2000). It has been shown that the progression of both DNA and RNA polymerases along the DNA is blocked at the site of platination, which results in the inhibition of replication and transcription processes (Todd and Lippard, 2009). If Pt-DNA adducts are not repaired, it will lead to cell death via an apoptotic pathway (Jung and Lippard, 2007).

Ionizing radiation modulates cancer cell response to the chemotherapeutic effect of Pt-drugs in several ways. Both Low-dose fractionated and hyper-fractionated radiation modify DNA repair capacity of a cancer cell that has been reported to enhance the
cytotoxicity of Pt-drugs, particularly in lung, head and neck cancers (Gupta et al., 2011; Jeremic et al., 2000). Radiation can also increase vascular permeability in the blood-brain barrier, leading to a greater accumulation of the drugs in central nervous system tumors (Cao et al., 2005). Moreover, radiation induces damage to cellular membrane, resulting in the enhancement of cellular uptake of the drugs (Yang, Douple and Wang, 1995). Both the increased vascular permeability and damage to cellular membrane prolong tumor retention of Pt-drugs and their effective concentration inside the cell (Spalding and Lawrence, 2006).

1.3. Molecular Basis of Radiation Therapy:

Radiotherapy is an effective and widespread method for treating cancer with curative, palliative and conservative purposes. A steady rise in the number of cancer patients results in increasing demand for radiotherapy services in Europe and North America. Roughly 45–55% of cancer patients require radiotherapy at some point and about 20–25% will have more than one course of radiation treatment (Connell and Hellman, 2009; Rosenblatt et al., 2013).

The main goal of physics-based technology in radiotherapy is to improve the ratio between an optimal radiation dose in the tumour tissue and the possible lowest dose in the healthy organs. Such an energy deposition of radiation in tumour and healthy tissues has serious biological consequences for the irradiated tissues and their cells, including lethal, sublethal, and potentially lethal damages that result in either local control or treatment of cancer and toxicity of health tissues as well. In addition to the technological improvements, the clinical practice of radiotherapy has been therefore influenced by biology underlying the responses of tumour and healthy cells to ionizing radiation (Connell and Hellman, 2009).

1.3.1. Biological Effects of Ionizing Radiation:

Ionizing radiation eradicates a malignant tissue via interaction with its cellular components. With respect to DNA as a main cellular target of radiation, the biological impact of ionizing radiation results predominantly from the formation of a variety of
lesions in DNA via energy deposition into the DNA itself (i.e., direct effect) and its surrounding molecular environment, particularly water molecules (i.e., indirect effect) (Goodhead, Thacker and Cox, 1993; O'Neill and Fielden, 1993; O'Neill and Wardman, 2009). The energy deposition generates intermediate species including ions, radicals, excited molecules and free electrons in a nanometer-scale volume. These species subsequently interact with DNA to induce cluster lesions (Goodhead, 2006; Ward, J.F., Webb C.F., Limoli C.L., Milligan J.R., 1990; Ward, 1994).

Owing to the considerable amount of water in a cell (about 70% of cellular mass), highly reactive species toward DNA arise from water radiolysis; the most importantly hydroxyl radicals (OH), hydrated electrons (e_{aq}^-) and hydrogen atom (H) (Clemens von Sonntag, 1987). It has been suggested that the indirect effect of radiation resulting from the water radiolysis has substantial contribution (i.e., 30 - 70 %) to the formation of DNA damage (DeLara, Jenner, Townsend, Marsden and O'Neill, 1995; Nikjoo et al., 2002). The reactive species induce a variety of DNA lesions such as base modifications, sugar damage leading to a base release and strand breaks (SSB and DSB), alkali-labile sites, and DNA-protein crosslink (Clemens von Sonntag, 2006). Among the various DNA lesions, strand breaks (particularly DSB) are believed to have considerable biological impact including lethality, mutagenesis and carcinogenesis (Negrini, Gorgoulis and Halazonetis, 2010; O'Driscoll and Jeggo, 2006; Obe, Johannes and Schulte-Frohlinde, 1992).

OH is the most reactive radical generated from radiolysis of water that interacts with DNA constituents at close to diffusion-controlled rate (Sevilla and Bernhard, 2008). Two main reactions of OH with nucleobases and sugar moiety are addition to C=C and C=N double bonds (e.g., formation of 8-oxoguanine) and hydrogen abstraction. OH has also been known as the main radical to induce DNA strand break via interaction with sugar moieties (e.g., hydrogen abstraction from C4' of sugar) (Clemens von Sonntag, 2006). In contrast, H and e_{aq}^- seems to be less potent at producing DNA damage, particularly strand breaks, despite the fact that they are highly reactive reducing species (Clemens von Sonntag, 1987; Li, Sevilla and Sanche, 2003). The observation of different
ionization potentials of $e^\text{aq}$ due to the localization of electron in the surface and inside (bulk) of water, however, suggest that the previous notion of $e^\text{aq}$ as a single equilibrated species in water seems to be too simple (Alizadeh and Sanche, 2012; Donald, Leib, O'Brien, Holm and Williams, 2008; Siefermann et al., 2010; Verlet, Bragg, Kammrath, Cheshnovsky and Neumark, 2005). Accordingly, experimental and theoretical studies report that $e^\text{aq}$, in particular at the surface of water can induce DNA damage, even strand breaks in the solvated DNA via electron transfer to a DNA subunits (e.g., phosphate group) and subsequent dissociation of a chemical bond (Nguyen, Maa, Luo, Bristow, Jaffray and Lu, 2011; Siefermann and Abel, 2011; Wang, Nguyen and Lu, 2009).

Although water represents a considerable fraction of cellular mass and the species arising from water radiolysis are highly reactive with DNA to induce a variety of lesions, DNA helix in a cell nucleus is known to be highly packed by forming a complex with histon protein (i.e., nucleosome) (Luger, Mäder, Richmond, Sargent and Richmond, 1997), which is further organized into a higher order chromatin structure in eukaryotic cells (Fig. 1.3) (Felsenfeld and Groudine, 2003). In this configuration, nuclear DNA, which includes a closely bound hydration shell with less than ~ 13 H$_2$O per nucleotide, is thus surrounded by a minimal amount of free water. This configuration reduces the indirect effects of radiation by protecting DNA from the diffusible radicals (Sevilla and Bernhard, 2008). Moreover, since hydroxyl radicals highly react with other biomolecules and its reaction rate with cellular components is close to diffusion-controlled rates, only those created in the immediate vicinity of DNA can induce damage (Clemens von Sonntag, 2006). Therefore, it should be recognized that intermediate species arising from the direct effect of radiation, including DNA subunit radical cations, free electrons and electronically excited DNA subunits, play a considerable role in the induction of DNA damage, particularly for irradiation with high linear energy transfer (LET) particles (Hirayama et al., 2009).
I.3.2. Interaction of Therapeutic Radiation with Biological Matter:
Despite various modalities in radiotherapy such as intensity modulated radiotherapy, tomotherapy, stereotactic radiotherapy, and brachytherapy, the types of radiation beam are essentially limited to photons including gamma- and X-rays, and charged particles including electrons and to much smaller extend protons and carbon ions (Thariat, Hannoun-Levi, Sun Myint, Vuong and Gérard, 2013). The dominant processes involved in the interaction of these ionizing radiations with biomolecules are ionization and excitation with the contribution of the energy deposited of 80% and 20%, respectively (Inokuti, 1995).

![Diagram of DNA packaging into chromatin structure](image)

Fig. I.3. Packaging DNA into the chromatin structure. (Reprint with permission from Macmillan Publishers Ltd: Nature (Felsenfeld et Groudine, 2003), Copyright 2003).
Photons interact with matter through five processes including Compton scattering, pair production, photoelectric effect, Rayleigh scattering and photonuclear interactions (Attix, 2004). Therapeutic photon beams of 0.2 – 20 MeV ionize biomolecules mainly through two types of interactions including Compton scattering and pair production. In the former, a photon interacts with a weakly bound (i.e., outer shell) electron of the scattering atom and transfers a fraction of its energy resulting in the ejection of the electron and scattering of the photon with lower energy. Such ejected electrons have a wide range of energy distribution, with a maximum energy less than the energy of interacting photons. Depending on the transferred energy, the scattered photon subsequently interacts with another molecule via one of the interaction processes. At energies less than 100 keV, while the interaction cross section for Compton scattering substantially decreases in biological matter, the photoelectric effect is the dominant interaction process. In this effect, the photon interacts with a tightly bound (i.e., inner shell) electron of the scattering atom and transfers its entire energy to the atomic electron. This photon vanishes and a photoelectron is ejected from one of the atomic inner shells. Similar to photoelectric effect, pair production is an absorption process in which the incident photon disappears and creates an electron and a positron, via the interaction with a strong Coulomb force field near an atomic nucleus. Obviously, the threshold energy required for the pair production occurring near a nuclear field is 1.02 MeV (i.e., sum of the rest mass energy of both electron and positron). In biological matter, however, its interaction cross section is only substantial for energies higher than 10 MeV. For photon energies in the range of 100 to 200 keV, Compton scattering is the predominant interaction process in biological media (Attix, 2004).

Charged particles lose their energy in a manner that is distinctly different from that of photons. Since a charged particle is surrounded by its Coulomb electric force field, it interacts with the atoms via two types of interactions: soft and hard collisions (Attix, 2004). When a charged particle passes an atom at a considerable distance, the impact of the particle’s Coulomb force field, which is termed soft collision, distorts the atom resulting in its excitation to a higher energy level, or ionization by ejecting a valence electron. When the distance between incident charged particle and an atom is of
the order of the atomic dimensions, hard collision occurs. In this case, the incident particle interacts primarily with a single atomic electron, which is then ejected from the atom with considerable kinetic energy. Although the probability of hard collisions is much less than that of soft collisions, the fraction of the energy transferred by incident particles is generally comparable for these two processes.

The energetic electrons, produced by Compton scattering, pair production and hard collision, have a wide energy distribution that interact with matter and they primarily lose their kinetic energy in a process similar to that of soft collisions. In this coulombic interaction, an incident charged particle transfers a very small amount of its kinetic energy and momentum to biological matter. Figure I.4(a) shows that the distribution of energy loss for energetic electrons interacting with DNA is mostly between a few eV and 100 eV with the mean energy loss at 23 eV (LaVerne and Pimblott, 1995). Owing to the ionization threshold of organic molecules, this amount of energy is capable of ionizing the molecules to produce cations and secondary electrons (SEs). Calculation of the energy distribution of SEs indicates that the vast majority of the electrons have energies below 30 eV and the most probable electron energy is between 9 and 10 eV. Fig I.4(b) shows the results of such a calculation for protons and He cations of different energies (Pimblott and LaVerne, 2007). Such a distribution is also independent of the mass of the fast charged particle, such that high-energy electromagnetic and any of charged-particle ionizing radiation produce such a distribution of SEs. Therefore, electrons of 0-30 eV, termed low-energy electrons (LEEs), are generated in copious number (i.e., on the order of $3 \times 10^{4}$ MeV of deposited energy) by any type of electromagnetic or charged-particle ionizing radiation and carry a substantial fraction of the energy of the primary ionizing radiation.

Low-energy secondary electrons can also be created via the Auger effect. When an electron is ejected from an inner atomic shell by the photoelectric effect or a hard collision, the formed excited state rapidly returns to the ground state via filling the electron vacancy by another electron from a less tightly bound shell. This process leads to
Fig. 1.4. (a) The calculated probability of energy loss in the soft collision of a charge particle with DNA, liquid water, gaseous water and gaseous hexane molecules, with no momentum transfer. The calculation is based on the dipole oscillator strength distribution [with permission from the Author, (LaVerne et Pimblott, 1995)]. (b) The energy distribution of secondary electrons generated by primary ions at different energies in water [with permission from the Author, (Pimblott et LaVerne, 2007)].

a cascade of electron transitions by which the atom exchanges one energetically deep inner shell vacancy for a number of relatively shallow outer shell vacancies. Conduction-band electrons finally neutralize these vacancies. Each electronic transition is accompanied by the emission of either a fluorescence X-ray photon or an Auger electron. Therefore, such an inner shell-ionized atom returns to its stable configuration by emitting a number of Auger electrons with a range of energies between a few eV and a few keV (Humm, JL. Howell, RW. Rao, DV, 1995; Nikjoo, Emfietzoglou and Charlton, 2008).

1.3.3. Interaction of LEEs with Condensed Matter:

When the electron wavelength is shorter than the atomic dimension (e.g., for moderate- and high-energy electrons), the electron can be considered to interact with the medium through individual localized processes, separated by mean free path much larger than the molecular dimension (Nikjoo, Emfietzoglou, Watanabe and Uehara, 2008). In contrast, LEEs have wavelengths comparable to intermolecular distances; hence they interact with solid and liquid media through delocalized processes predominantly including static and correlation interactions with neighbouring molecules (i.e., distortion of the bound electron density of neighbouring molecules in the presence of a field of the
incident electron, resulting in electronic, vibrational, and/or rotational excitations), excitation transfer (i.e., energy transfer of an excited state of a molecule to a neighboring molecule, leading to the excitation of that molecule), and coherent scattering. These processes mainly result from two types of force between an incoming electron and a target molecule: (1) the induced polarization attraction which distorts the target orbitals, and (2) the exchange force which describes the electron-target system wave function (Carsky and Curik, 2012). At these low energies, particularly less than 15 eV, there is a strong probability that one or more partial waves of the electron wave function undergo constructive interference (i.e., resonance) within the scattering molecule (Sanche, 1991). Therefore, LEE-molecule interaction can be described by resonant and nonresonant (direct) phenomena.

I.3.3.1 Resonant Process:

Electron resonances occur when an incoming electron is temporarily captured into an unfilled orbital of a molecule for a time longer than the usual scattering time. Therefore, the resonant processes involve the formation of a transient negative ion (TNI) with a survival time between $10^{-15}$ and $10^{-3}$ s. There are two major types of TNIs or electron resonances: shape and core-excited resonances (Schulz, 1973). When the captured electron in a TNI occupies a previously unfilled orbital in its ground state, the transitory state is referred to as a single-particle or shape resonance. The energy level of this type of resonances lies above their parents. In this case, the electron-molecule potential forms a penetrable barrier due to the angular momentum from the nonzero momentum partial wave content of the incoming electron, that is responsible for the temporary trapping of the electron near the molecule. The term “shape” represents the shape of the potential that is responsible for the electron trapping. If a TNI is formed by two electrons occupying previously unfilled orbitals, the transitory state is called a “core-excited” or two-particle, one-hole resonance. Such resonances can lie slightly below or above the energy of the electronically excited state of its neutral parent. In the latter case, a momentum barrier (i.e., a penetrable barrier resulted from the angular momentum) in the electron-molecule potential, similar to the shape resonance, contributes to retain the electron in the molecule; hence it is referred as core-excited shape resonance (Sanche,
In the former case, the incoming electron is essentially captured by the positive electron affinity of an electronically excited state of the molecule. Since such a TNI falls in the category of Feshbach resonances, they usually have a relatively long lifetime of the order of $10^{-12}$ to $10^{-14}$ s. Such a long lifetime entails a substantial increase in the interaction time between an incoming electron and the scattering molecule, which results in an enhancement of inelastic cross sections by orders of magnitude (Caron and Sanche, 2012).

Decay channels of a molecular TNI are usually more than its atomic counterpart since it has more degrees of freedom. Dissociative electron attachment (DEA), electron autodetachment, autoionization and resonance stabilization are the four possible decay channels of a TNI (Sanche, 1991). 

DEA will result in the dissociation of a TNI, if the TNI has three conditions: 1) dissociative state accessible in the Frank-Condon region, 2) resonance lifetime longer than about half a vibration period of the anion (Sanche, 2012), and 3) positive electron affinity for one of the possible fragments. Under these conditions, one or several bonds of the TNI dissociate via a DEA decay channel to produce a stable anion and a neutral atomic or molecular species in the ground (reaction 1) or an excited state (reaction 2):

$$RH + e^- \rightarrow (RH)^- \rightarrow R + H^- \quad \text{(or } R^- + H), \quad \text{Reaction 1}$$

$$RH + e^- \rightarrow (RH)^{*-} \rightarrow R* + H^- \quad \text{(or } R^- + H*). \quad \text{Reaction 2}$$

Electron autodetachment or autoionization (of the anion) results in a departing electron with lower kinetic energy and a neutral molecule in a rotationally, vibrationally and/or electronically excited state:

$$RH + e^- \rightarrow (RH)^{*-} \rightarrow (RH)^* + e^- . \quad \text{Reaction 3}$$

Depending on the electronically excited state (i.e., dissociative or non-dissociative) and its energy (i.e., transferred from the previously attached electron), the excited state may autodissociate into neutral or ionic fragments:
When the parent molecule of a TNI has a positive electron affinity, if the TNI in an excited state can transfer its energy to another molecule or molecules, it can become a permanent stable anion. This phenomenon is called charge stabilization:

\[
(RH)^* \rightarrow R + H \quad \text{(or } R^- + H^+) \quad \text{Reaction 4}
\]

\[
RH + e^- \rightarrow (RH)^* \rightarrow (RH)^- + \Delta E \quad \text{Reaction 5}
\]

Autoionization can also lead to cation formation when two electrons autodetach from the TNI. In this case, we have:

\[
RH + e^- \rightarrow (RH)^- \rightarrow (RH)^+ + 2e^- \quad \text{Reaction 6}
\]

1.3.3.2. Non-resonant Process:

The non-resonant interactions of an incoming electron and a scattering molecule mainly result in the ionization and excitation (i.e., rotational, vibrational and/or electronic excitation states) processes. Interaction cross sections for this type of scattering, in contrast to the resonances, change slowly with incident electron energy. The curves indicating the yield of molecular fragments resulting from non-resonant electron impact (i.e., yield functions) are usually distinguished by a smooth and monotonically rising signal above a threshold and a broad peak at higher energies (Fig. 1.5.a, the curves shown by dashed lines) (Huels, Boudaiffa, Cloutier, Hunting and Sanche, 2003). The threshold is the energy required for the dissociation of a neutral excited intermediate state formed via non-resonant interaction:

\[
RH + e^-(E_0) \rightarrow (RH)^* + e^-(E) \quad (E < E_0). \quad \text{Reaction 7}
\]

Such threshold energy corresponds to the lowest dissociation energy of \((RH)^*\) which may produce neutral or ionized fragments:
(RH)* → R + H  (or  R⁺ + H⁻).  

The dissociation energy results from the electrodynamics interaction potential between a scattering molecule and an incident electron outside the molecule. The potential is mainly made of electrostatic force (e.g., dipole and quadrupole moments) and polarization attraction (i.e., the dynamic interaction) induced by the incident electron (Sanche, 1991).

At higher energies of the incident electron, ionization is the main process, which results in the formation of non-dissociative or dissociative cations:

\[ RH + e^- \rightarrow (RH)^* + 2e^-, \]  

\[ RH + e^- \rightarrow (RH)^{**} + 2e^-. \]  

Similar to neutral excited state, the excited cation may dissociate:

\[ (RH)^* \rightarrow R + H^*  \quad \text{(or} \quad R^+ + H). \]

1.3.4. Interaction of LEEs with DNA:

Cellular DNA is composed of two antiparallel polynucleotide strands that twist into a double helix. The backbone of the strand consists of repeating deoxyribose molecules and phosphate groups that are covalently linked to each other by phosphodiester bonds. Each deoxyribose molecule in the backbone is covalently attached to a heterocyclic base as a side chain by a glycosidic bond. The bases are purines and pyrimidines. The former are adenine and guanine, and the latter are cytosine and thymine. The bases of one strand are paired with those of the opposite strand by hydrogen bonds, so that an adenine is always paired with a thymine, while a cytosine is always paired with a guanine.

LEEs induce a variety of lesions in DNA by the rupture of chemical bonds between the DNA subunits, including the breakage of phosphodiester and glycosidic bonds resulting in strand breaks (i.e., SSB and DSB) and base release, respectively.
While both resonant and non-resonant processes are able of inducing these types of DNA damages, their contribution highly depends on the incident electron energy. For electron energies below 15 eV, the yield functions for the formation of strand break depend strongly on the incident electron energy, whereas at higher energies the major portion of the yields monotonically rises and saturates above 50 eV (Huels, Boudaïffa, Cloutier, Hunting and Sanche, 2003). These behaviours indicate that electron resonances are the main mechanisms responsible for the electron damage to DNA at energies less than 15 eV, while non-resonant scattering results in the formation of damage at higher energy, particularly above 40 eV. Between 15 and 40 eV, there are broad resonances that are superimposed on the nonresonant scattering background. The maximal peaks for the formation of SSB by LEEs have been observed at the electron energies of 10, 2.2 and 0.8 eV, while for the DSB the corresponding peak is only at 10 eV (Boudaïffa, Cloutier, Hunting, Huels and Sanche, 2000; Martin, Burrow, Cai, Cloutier, Hunting and Sanche, 2004). Fig. 1.5 shows that the maximal yields for SSB and DSB at these resonant energies have similar magnitude to those of 100 eV electrons (i.e., an energy at which electrons have the highest cross section to damage molecules) principally due to their high

![Graph](image)

ionization yield. This finding suggests that the probability of the formation of strand break per incident electron is similar for both resonant and nonresonant processes, whereas the incident electron energies for resonances are one to two orders of magnitude smaller than those for non-resonant scattering (Sanche, 2009a).

Induction of strand breaks by 10-eV electrons has been described by the initial formation of core-excited TNI of specific DNA subunit, predominantly a base or phosphate group, and decaying into DEA and/or dissociative electron excitation channels. At 0.8 and 2.2 eV, the electrons energies are below the electronic excitation of organic molecules; hence strand breaks are essentially formed through the shape resonances of a basic DNA constituent and its decay into DEA.

1.4. Radiosensitization Effects of Platinum Chemotherapeutic Drugs:

Several potential mechanisms are proposed for cellular and molecular radiosensitization mediated by Pt-drugs including (Dewit, 1987; Seiwert, Salama and Vokes, 2007; Wilson, Bentzen and Harari, 2006):

1- *Increased reactive species:* Reaction of radiation-induced free radicals with platinum atom of Pt-drugs can generate highly reactive intermediate species (Richmond, 1984; Swancutt, Mezyk and Kiddle, 2007). Since platinum is a metal with the ability of having various oxidation states, they can undergo oxidation-reduction reactions in cellular conditions and subsequently generate reactive oxygen species (ROS) and additional 'OH via a Fenton-type reaction. Both hydrogen peroxide (H$_2$O$_2$) and 'OH can oxidize Pt-drugs (i.e., Pt$^{II}$ molecules) to Pt$^{III}$ species. For cisplatin, this could be:

\[
(NH_3)_2Pt^{II}Cl_x(H_2O)_y + H_2O_2 \rightarrow (NH_3)_2Pt^{III}Cl_x(H_2O)_y + HO^- + HO^+ \]  \hspace{1cm} \text{Reaction 12}
\[
(NH_3)_2Pt^{II}Cl_x(H_2O)_y + HO^- \rightarrow (NH_3)_2Pt^{III}Cl_x(H_2O)_y + HO^- \]  \hspace{1cm} \text{Reaction 13}

where $x$ and $y$ are 0, 1 or 2 and $x$ plus $y$ equals 2. The Pt$^{III}$ species can react with dissolved molecular oxygen to produce superoxide radical anions:
Pt-drugs may also react with hydrated electrons to reduce Pt\textsuperscript{I} species, which can subsequently react with molecular oxygen to generate superoxide radical anions:

\[
\left(\text{NH}_3\right)_2\text{Pt}^{\text{III}}\text{Cl}_x\left(H_2O\right)_y + O_2 \rightarrow \left(\text{NH}_3\right)_2\text{Pt}^{\text{IV}}\text{Cl}_x\left(H_2O\right)_y + O_2^- \quad \text{Reaction 14}
\]

It is also reported that weakly-bound electrons localized at the surface of water cluster (i.e., prehydrated electron) can transfer to cisplatin molecule, release the chlorine anion via DEA and generate a reactive radical of cisplatin, which can subsequently induce damage to cellular components such as DNA (Kuduk-Jaworska, Chojnacki and Jański, 2011; Lu, 2007; Lu, Kalantari and Wang, 2007):

\[
\left(\text{NH}_3\right)_2\text{PtCl}_2 + e_{aq}^- \rightarrow \left[\left(\text{NH}_3\right)_2\text{PtCl}_2\right]^- \rightarrow \left(\text{NH}_3\right)_2\text{PtCl}^{\cdot} + Cl^- \quad \text{Reaction 17}
\]

The presence of NH\textsubscript{3} groups in the cisplatin molecule increases the polarity of the molecule that is suggested to favor the transfer of prehydrated electrons and the formation of TNI at the cisplatin.

2- Enhanced Pt-drugs uptake: Ionizing radiations have been suggested to increase the cellular uptake of Pt-drugs. In-vitro studies have shown that cellular uptake of carboplatin increases by factors between 1.3 and 1.6 in cultured V79 cells and in Chinese hamster ovary (CHO) cell lines due to radiation-induced damage to cellular membrane (Yang, Douple and Wang, 1995).

3- Inhibition of radiation-induced damage to DNA: Pt-drugs have been shown to inhibit sublethal and potentially lethal damage repair (SLDR and PLDR) induced by ionizing radiation in mammalian cells (Bergs, Franken, Ten Cate, Van Bree and Haveman, 2006; Caney, Singh, Lukka and Rainbow, 2004). These types of DNA damage occur after a cell is exposed to a radiation dose insufficient to cause cell death. The cell is able to repair the
damages under the physiological (i.e., SLDR) or a particular condition (i.e., PLDR) and recover itself to full mitotic potential. Pt-drugs can inhibit SLDR and PLDR by forming Pt-adducts, which possibly impede the enzymatic corrections of radiation-induced damage to the platinitated DNA (Bergs, Franken, Ten Cate, Van Bree and Haveman, 2006). Cisplatin, for example, is reported to inhibit non-homologous end-joining (NHEJ) repair by reducing the translocation of Ku proteins on the broken end of DNA (Boeckman, Trego and Turchi, 2005; Myint, Ng and Raaphorst, 2002). NHEJ is one of the important pathways to repair DNA DSBs and Ku proteins plays a vital role in NHEJ to protect the broken ends of DNA from degradation, bridge the ends before joining, recruit and activate repair proteins, particularly the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs).

4- Increased radiation-damage to DNA: Since the platinum atom is a heavy metal element with high atomic number (i.e., 78), it has large cross section for the interaction with photons via photoelectric effect. When photon energy corresponds to the absorption edge of the inner shells of platinum atom, the photons ionize the platinum atom and can induce the emission of Auger electrons. Both cisplatin and chloroterpyridine platinum (PtTC) placed in the close vicinity of DNA have been reported to enhance the formation of DNA strand breaks and cellular toxicity by monoenergetic synchroton X-rays at the K-shell edge of platinum atom, owing to the generation of numerous low energy Auger electrons which subsequently transfer their energy within DNA and induce cluster lesions (Biston, Joubert, Charvet, Balosso and Foray, 2009; Kobayashi, Usami, Porcel, Lacombe and Le Sech, 2010). However, this mechanism is less probable in the routine radiotherapy with high-energy photons and electrons since such radiations have energies much higher than the optimal energy for inner-shell ionization of platinum atom. These clinical radiation beams interact with matter mostly via Compton effects and/or soft collisions that predominantly result in outer-shell ionization (Attix, 2004).

Another important characteristic of the platinum atom associated with radiosensitization is its high electron affinity which has been suggested to result in a higher cross section for DNA damage induced by LEEs (Sanche, 2009b). It has been
reported that cisplatin enhances the formation of SSB and DSB by LEEs in a solid film of plasmid DNA (Zheng, Hunting, Ayotte and Sanche, 2008).

1.5. Research Project:

Concomitant chemoradiation therapy based on the platinum anticancer drugs (i.e., cisplatin, carboplatin and oxaliplatin) improves the treatment of several solid tumors by enhancing local therapy. Since DNA is the primary target of both radiation and Pt-drugs, radiosensitization effects of Pt-drugs results essentially from their binding to the nuclear DNA. One of the suggested mechanisms is the sensitization of DNA towards radiation by Pt-drugs, which leads to increase in the radiation-induced damage to DNA. Since ionizing radiations induce DNA lesions via both the direct and indirect effects, knowledge about the contribution of each effect in the enhanced radiation damage to DNA modified by Pt-drugs is useful, particularly for selecting the type of radiation beam (e.g., low or high LET) for the CCRT based on Pt-drugs. For the direct effect, LEEs are the most abundant of secondary species that can induce a variety of lesions in DNA via resonant processes. The presence of atoms with high electron affinity such as halogen atoms within the interacting molecule has been shown to substantially enhance DEA channel, particularly in the presence of polar molecules such as NH$_3$, resulting in the dissociation of the molecule (Faradzhev, Perry, Kusmierek, Fairbrother and Madey, 2004; Lu and Sanche, 2001). Pt-drugs contain both polar molecules and platinum, which has high electron affinity (i.e., 2.13 ± 0.002 eV), hence Pt-DNA adducts are hypothesized to highly sensitize DNA towards LEEs.

In the chemotherapeutic implication, carboplatin is an appropriate alternative to cisplatin due to its lower side effects and its spectrum of activity similar to cisplatin. Such information about radiosensitization efficiency of Pt-drugs is useful for the optimization of protocols in CCRT. However, such knowledge is not available in the literature. Therefore, this research project includes four objectives:

1- to determine the contribution and efficiency of LEEs in the induction of DNA damage by direct effects of ionizing radiation in the presence of Pt-drugs,
INTRODUCTION

2- to compare the relative efficiency of Pt-drugs for DNA sensitization towards the direct effect of radiation, in terms of the type of Pt-drug (i.e., cisplatin, carboplatin and oxaliplatin) and the quantity of Pt-DNA adducts.

3- to determine the major mechanisms responsible for the enhanced radiation damage to DNA modified by Pt-drugs, and

4- to determine the contribution and efficiency of \( ^{\cdot}\text{OH} \) and \( e_{\text{aq}}^- \) in the induction of damage to platinated DNA by the indirect effect of ionizing radiation.

To investigate these objectives, supercoiled plasmid DNA was chosen as an appropriate model of DNA. Such a plasmid DNA in combination with agarose gel electrophoresis is a popular and useful method for determining DNA lesions, particularly strand breaks in a cell-free system based on the topological changes in the DNA (Hempel and Mildenberger, 1987). By this method, a wealth of information on the mechanisms of DNA strand breaks induced by different types of radiation has been understood (Boudaïffa, Cloutier, Hunting, Huels and Sanche, 2000; Folkard and Prise, 2006; Hunniford, Timson, Davies and McCullough, 2007; Kobayashi, Usami, Porcel, Lacombe and Le Sech, 2010; Purkavastha, Milligan and Bernhard, 2005). Since both plasmid DNA and eukaryotic DNA are negatively supercoiled, the conformational distortion of DNA resulting from the formation of Pt-adducts should be similar in the both types of DNA. Such distortion in DNA structure is proposed to influence radiation damage to DNA modified by Pt-drugs (Sanche, 2012). Unfortunately, the available methods published in literature for the preparation of Pt-drug bound to plasmid DNA (i.e., Pt-DNA complex) were not appropriate for this study, since the conditions for the reaction of DNA platination used in these methods highly sensitize plasmid DNA to any further sample manipulation. In this research project, therefore, a new method has been developed for the preparation of Pt-DNA samples which is reported in first manuscript:

**Article No. 1:** *DNA-platinum thin films for use in chemoradiation therapy studies.*

This article introduces a new method for the preparation of Pt-DNA samples in both solution and thin film, which the latter is essential for the study of the direct effect of radiation, particularly LEEs on DNA modified by Pt-drugs. By this method, it is possible
to maintain the DNA integrity during the reaction of DNA platination and to control the number of Pt-DNA adducts.

To determine the first objective, it was essential to compare the yields of DNA damage induced by LEEs with those by high-energy electrons. Since DNA damage is resulted from the energy deposited in the samples, the yield of damage should be obtained based on the absorbed dose. Unfortunately, the yields of DNA damage by LEEs reported in literatures were only obtained based on the number of damage per incident electron. In addition, these yields highly depended on the experimental conditions, mainly including film thickness and charging effect. Therefore, a new mathematical method has been developed in this research project to overcome these obstacles, which resulted in Article No. 2:

**Article No. 2:** Absolute cross section for low-energy-electron damage to condensed macromolecules: A case study of DNA.

This article introduces a mathematical model, termed a molecular survival model, to transform a measured effective cross section for DNA damage induced by LEEs into an absolute cross section independent of experimental conditions. By this model, it is possible to extract the essential parameters (e.g., attenuation length) required for the calculation of absorbed dose, from experimental data obtained in the LEE irradiation of a solid film of DNA.

The first three objectives resulted in two manuscripts:

**Article No. 3:** New insights into the mechanism underlying the synergistic action of ionizing radiation with platinum chemotherapeutic drugs: The role of low energy electrons.

**Article No. 4:** A single subexcitation-energy electron can induce a double strand break in DNA modified by platinum chemotherapeutic drugs.

The last objective resulted in the **Article No. 5:** Cisplatin enhances the formation of DNA single-and double-strand breaks by hydrated electrons and hydroxyl radicals.
II. RESULTS - ARTICLES

II.1. First Article: DNA-Platinum Thin Films for Use in Chemoradiation Therapy Studies

Authors: Mohammad Rezaee, Elahe Alizade, Darel J. Hunting, Léon Sanche

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Contributions: Mohammad Rezaee designed and performed research, analyzed data and wrote manuscript. Professor Leon Sanche and Professor Darel Hunting supervised the project, advised on the data interpretation and edited the manuscript. Dr. Elahe Alizadeh advised on nanoscale film preparation and data analysis.
DNA-Platinum Thin Films for Use in Chemoradiation Therapy Studies

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ABSTRACT
Dry films of platinum chemotherapeutic drugs covalently bound to plasmid DNA (Pt-DNA), represent a useful experimental model to investigate direct effects of radiation on DNA in close proximity to platinum chemotherapeutic agents, a situation of considerable relevance to understand the mechanisms underlying concomitant chemoradiation therapy. In the present study we determine the optimum conditions for preparation of Pt-DNA films for use in irradiation experiments. Incubation conditions for DNA platination reactions have a substantial effect on the structure of Pt-DNA in the films. The quantity of Pt bound to DNA as a function of incubation time and temperature is measured by inductively coupled plasma mass spectroscopy. Our experiments indicate that chemical instability and damage to DNA in Pt-DNA samples increases when DNA platination occurs at 37 °C for 24 hours, the condition which has been extensively used for in-vitro studies. Platination of DNA for the formation of Pt-DNA films is optimal at room temperature for reaction times less than 2 hours. By increasing the concentration of Pt-compounds relative to DNA and thus accelerating the rate of their mutual binding, it is possible to prepare Pt-DNA samples containing known concentrations of Pt while reducing DNA degradation caused by more lengthy procedures.

INTRODUCTION
Clinical studies have shown that concomitant treatment with chemotherapeutic drugs and radiotherapy often leads to a higher rate of survival and local tumor control compared to non-synchronous treatments [1, 2]. Platinum chemotherapeutic drugs are commonly used in concurrent chemoradiation therapy (CRT) for treatment of solid tumors [3]. Although it is clear that platinum drugs and radiation in CRT modalities increases tumor cell killing, improves locoregional control of tumors and enhances patient survival [4, 5], the optimum schedule of the combination and the underlying mechanisms of their synergistic action have not yet been defined [6, 7]. Since DNA is the common target of both radiation and platinum chemotherapeutic agents, most studies have focused on the structural and functional alteration of DNA resulting from the combination [8, 9]. One possible mechanism responsible for the observed synergy is
enhancement in immediate (secondary) species induced by primary radiation in the vicinity of the binding site of the platinum compounds (Pt-compounds) to DNA [10, 11]. The most abundant of these secondary species are electrons with the most probable energy of 9–10 eV [12]. Studies on the interaction of secondary low energy electrons (LEEs) with DNA have elucidated some of the fundamental mechanisms leading to DNA damage [13]. However, owing to the short range (~ 10 nm) of LEE in biological matters, such studies must be performed on very thin DNA films of similar thickness. Pt-DNA thin films could provide an experimental approach to investigate the direct effects of the secondary electrons and other short-range particles (or secondary species) on DNA in the presence of Pt-compounds. Such investigations could disclose mechanisms underlying the synergistic effect between the radiation and the drug, which may have implications for the optimization of protocols in CRT as well as in the design and development of new chemotherapeutic and radiosensitizing drugs [14].

Dry thin films of bacterial plasmid DNA in supercoiled conformation are widely used in low-energy irradiations with LEEs [15, 16], photons [17], and ions [18]. They provide a simple system to evaluate the direct interaction of short-range radiations with DNA, despite the complexity of the molecule. Although purified prokaryotic DNA differs from eukaryotic DNA in terms of supercoiling and presence of N6-methyladenine [19, 20], supercoiled plasmid DNA offers the advantage of very high sensitivity for the detection of single and double strand breaks. One of the main concerns with plasmid DNA films is maintenance of the DNA integrity during film preparation [21]. When the irradiation target is supercoiled DNA, the proportion of the supercoiled configuration is often used as a measure of DNA integrity. The DNA molecule is very sensitive to conditions such as temperature, humidity, pH, etc. and hence, the DNA films must be prepared under well-controlled conditions to minimize damage. The concentration of ions in solution of DNA has also a considerable influence in maintaining the DNA during film preparation [21, 22]. Furthermore, the type of substrate on which DNA is deposited affects the integrity of the molecule. Among the various substrates tested including gold, graphite, etc., tantalum (Ta) induces the least damage to DNA [23].

Pt-compounds such as cisplatin and carboplatin bind to the N7 atom of purine bases and produce the Pt-DNA adducts including mainly intrastrand cross-links,
interstrand cross-links, and monofunctional binding to guanine [24]. The adducts distort the DNA conformation and reduce the structural stability of DNA [24, 25]. Moreover, DNA must tolerate the incubation conditions required to react with Pt-compounds. In most in-vitro studies, a DNA solution is mixed with a solution of the Pt-compounds at 37 °C for 24 or 48 hours [26 - 30]. These conditions affect the integrity of the DNA as a result of depurination and oxidation processes [31]. To maximize the amount of the Pt-compounds bound to DNA while keeping the DNA intact, all parameters involved in the preparation of the films must be known and carefully controlled. In particular, experimental conditions for the reaction of Pt-compounds with DNA must be determined as well as the effect of chemical binding of Pt-compounds on the stability of DNA.

In the present study, we investigate the parameters of the Pt-compounds and platination reactions on DNA integrity in the preparation of cisplatin/DNA and carboplatin/DNA films. Optimum experimental conditions are determined to retain a high proportion of the supercoiled form of plasmid DNA in Pt-DNA films.

EXPERIMENTAL SECTION

Preparation of Plasmid DNA. Plasmid DNA (pGEM-3Zf(-), 3197 base pairs, ca. 1968966 amu per plasmid) was extracted from Escherichia coli JM109 and purified with a HiSpeed plasmid Maxi kit (QIAGEN) [32]. The purified plasmid DNA consisted of 96% supercoiled, 2% concatemeric, and 2% nicked circular forms. The concentration of DNA and the relative quantity of proteins in the plasmid DNA solution was then calculated by measuring the ratio of ultraviolet (UV) absorption of DNA and protein at 260 nm and 280 nm, respectively, with a Synergy HT-I spectrophotometer. The ratio was 1.98 which corresponds to a purity greater than 85% [33]. The TE buffer (Tris-EDTA: 10 mM–1 mM) was separated from DNA by gel filtration with a Sephadex G-50 medium [34]. Thus the final solution consisted of DNA and ddH2O after the filtration. To evaluate the effect of Tris on the binding of Pt-compounds to DNA, two different groups of the DNA solutions were prepared. In the first group, Tris buffer was added to the DNA solution at the ratio of the one tris compound per nucleotide, and in the second group, the DNA solution was prepared with ddH2O alone. The DNA concentration was the same in
both groups. In each group, control samples were kept in the temperature of -20 °C and quantified for the analysis of temperature effect on DNA.

**Platination of Plasmid DNA.** The Pt-compounds, cisplatin [cis-diamminedicloroplatinum(II)] and carboplatin [cis-diammine(1,1-cyclobutane-dicarboxylato)platinum(II)], were purchased from Sigma-Aldrich with a stated purity of 99.9% and ≥ 98%, respectively, and used without further purification. Their solutions were prepared in ddH₂O in different concentrations based on their molar solubility. Reactions of cisplatin and carboplatin with the DNA solutions were performed under diverse experimental conditions. These consisted of (1) two different incubation temperatures, i.e. 37 °C and 25 °C, (2) incubation times varying from 40 minutes to 24 hours, and (3) molar ratios between Pt-compounds and DNA varying from ratios 2:1 up to 200:1. DNA platination reactions were performed in the dark to inhibit photoaquation processes as aqueous solutions of cisplatin and carboplatin are degraded via illumination, especially at wavelengths below 500 nm [35, 36]. To terminate the reactions after a given incubation time, the solutions were passed through a gel filtration medium packed into a column. By the filtration, the unbound Pt-compounds, tris compounds, and complexes of tris with Pt-compounds, were separated from the Pt-DNA solutions. The solutions passed through the homemade column packed with Sephadex-G50 gel on a glass bead bed. Sephadex G-50 is a suitable medium for separation of the molecules having a molecular weight larger than $3 \times 10^4$ g mol⁻¹ from molecules with a molecular weight smaller than 1500 g mol⁻¹. Such filtration is expected to produce clean solutions of Pt-DNA in ddH₂O because the molecular weights of most undesired compounds and complexes found in the solutions during platination have the molecular weight smaller than 1500 g mol⁻¹.

**Analysis of Platinum-DNA Binding.** The concentration of platinum in the solutions was measured by Elan DRC II inductively coupled plasma mass spectroscopy (ICP-MS, from Perkin Elmer) which has been used as a suitable method for measurement of platinum in many biomedical applications [37, 38]. Additionally, three control samples consisting of the Pt-compounds dissolved in ddH₂O at known concentrations were also prepared to calibrate the ICP-MS measurements of Pt-DNA samples. The DNA concentration was measured by spectrophotometry. It was determined from the optical density of DNA in
solution measured by UV absorption at a wavelength of 260 nm. The concentration of DNA was calculated from the reference optical density.

**Preparation of Substrate, DNA, and Pt-DNA Films.** The DNA and Pt-DNA samples were deposited on a Ta substrate. As shown in previous studies, the stability of supercoiled plasmid DNA on Ta substrate is acceptable for vacuum experiments on LEE-induced damage [23, 39]. The Ta substrates in the current work consist of a thin film of Ta of thickness 450 ± 50 nm evaporated onto a 0.4 mm thick silicon wafer. The surface of Ta was cleaned in pure ethanol and ddH2O and dried with a flow of dry nitrogen. Before deposition of DNA and Pt-DNA samples onto the substrate, the TE buffer was added to the DNA and Pt-DNA solutions in the ratio of 3:1 (three organic ions per nucleotide). It has been shown that this ratio protects the supercoiled form of DNA during the process of DNA film preparation [22]. The volumes of 7 µl of the latter solutions of DNA and Pt-DNA consisting of 250 ng of each complex (DNA and TE molecules as well as Pt-DNA and TE molecules) were deposited onto the cleaned Ta surface. These quantities were calculated to allow formation of a five monolayer film (about 10 nm thickness) on the Ta substrate. Such a thickness has been widely used in DNA-LEE experiments because it is smaller than the effective range of these electrons (12–14 nm) for damaging DNA [40]. After freezing at −65 °C for 10 minutes in a glove box, the samples were lyophilized (freeze-dried) under a pressure of 7 mTorr by a hydrocarbon-free turbomolecular pump for 2 hours.

**Quantification of the DNA and Pt-DNA Films.** The DNA and Pt-DNA films were recovered from the Ta substrates with 10 µl of TE buffer. Comparison of the amount of recovered DNA with the original DNA solution used for deposition showed that approximately 98% of DNA was recovered by the TE buffer. Quantification of the different structural forms (e.g. supercoiled, nicked circular, linear, etc.) in the DNA and Pt-DNA samples was performed by agarose gel electrophoresis. The DNA samples and the agarose gels were stained with SYBR Green I in the concentration of 100X and 10000X, respectively. The samples were run on 1% agarose gel in 1X TAE buffer at 100 volts for 7 minutes following by 75 volts for 68 minutes (5 V cm⁻¹). The gels were then scanned by Typhoon-Trio laser scanner (from GE Healthcare) adjusted for the blue
fluorescent mode at an excitation wavelength of 488 nm and filter type 520 nm-bandpass (520 BP 40) in the normal sensitivity mode. Various forms of the DNA such as supercoiled, nicked circular, etc. were analyzed by ImageQuant 5.0 (Molecular Dynamics) software. To accurately quantify, the binding efficiencies of SYBR Green I for the same amount (75 ng) of supercoiled and linear DNA were measured and then the correction factor was determined. This factor arises from the weaker binding of SYBR Green I to supercoiled DNA than to the nicked circular and linear forms. A correction factor of 1.2 was obtained and applied to the quantification of plasmid DNA.

Statistical Analysis. OriginPro 8.1 SR1 (OriginLab Corporation) software was used for statistical and mathematical analysis. Paired t-Test was the statistical test in which a probability of 0.05 (5%) has been considered significant.

RESULTS AND DISCUSSION

Effects of Incubation Temperature on DNA and Pt-DNA Samples. Figure 1 (panels a and b) shows a comparison of the percentage of supercoiled and nicked circular forms of the DNA in the samples that had been incubated for 24 hours at three different temperatures: -20°C, 25°C, and 37°C. For each incubation temperature, DNA analysis was performed for two types of samples: i) “DNA solutions” i.e., samples obtained directly from the incubated solutions, and ii) “DNA films” i.e., samples that had, after incubation, been deposited onto- and recovered from a Ta substrate. TE buffer was added to the samples at a concentration corresponding to three organic ions per nucleotide. Increasing the incubation temperature resulted in a reduction of the supercoiled form of DNA in both the solution and the film samples. The decrease is relatively small for the DNA solution samples; the samples incubated at 25 °C and 37 °C show a decrease of 3.8% and 9.5%, respectively, relative to that seen in the sample maintained at -20 °C. At each temperature, the DNA samples recovered from Ta show a greater loss of supercoiled DNA than do the samples analyzed directly from solution. A fraction of the supercoiled loss in the film samples is related to the damages which were induced during the incubation in solution. Consequently, for DNA recovered from Ta, a decrease in the supercoiled form with increasing temperature is also observed, and the decrease is very
Figure 1 Comparison of the percentages of DNA supercoiled (a), DNA nicked circular (b) and Pt-DNA supercoiled (c) forms in the solutions and films samples after incubation at -20 °C, 25 °C, and 37 °C for 24 hours. Data in a–c are means from three independent experiments; three samples at each temperature are analyzed in each experiment; error bars show standard deviations.

* indicates p-value > 0.05

** indicates p-value < 0.05
large for the samples incubated at 37 °C. Considering pair t-test with the probability value of 0.05 as a level of significance, the decreases in the supercoiled form are not statistically significant among the DNA solution samples with different incubation temperatures (p-value: 0.314, 0.106). However, the difference is statistically significant between the DNA film samples incubated at 37 °C and the DNA films from samples incubated at 25 °C and -20 °C (p-value: 0.012 and 0.009). Additionally, there is no significant difference between the DNA films incubated at 25 °C and -20 °C (p-value: 0.136).

As expected, there are enhancements in the formation of the nicked circular form with increasing incubation temperature. The increase is small except for the DNA film samples which were incubated at 37 °C. In these samples the nicked circular form increases by factors of 3.7 and 3.4 compared to those kept at -20 °C and 25 °C, respectively. These differences are statistically significant (p-value: 0.02 and 0.011). The high proportion of the nicked circular form in the DNA recovered from films introduces considerable inaccuracy in the evaluation of radiation induced DNA damage.

In-vitro studies have shown that heat can induce various types of DNA damage such as depurination and guanine oxidation mediated by reactive oxygen species (ROS) [31, 41]. Reaction rate constants for formation of 8-oxoguanine and guanine depurination at 37 °C are $4.7 \times 10^{-10}$ s$^{-1}$ and $1.3 \times 10^{-9}$ s$^{-1}$ in DNA solutions, respectively [41]. In our experiment, each plasmid sample contained 0.065 pmole of DNA bases in a volume of 7 μL. After a 24 hour incubation of the plasmid DNA at 37 °C, we can estimate that approximately 7% and 18% of the plasmid contain 8-oxoguanine molecules or have undergone guanine depurination, respectively. Such DNA molecules are more susceptible to strand breakage than the original DNA. Furthermore, evacuation and lyophilisation during film preparation induce physical stress and can damage DNA [21]. Therefore, the DNA molecules which have been kept at 37 °C for 24 hours or more, do not have sufficient structural stability to tolerate the process of film preparation. Our results suggest that the samples incubated at 37 °C are more sensitive and vulnerable to the film preparation and recovery processes than DNA samples incubated at 25 °C and -20 °C.

Figure 1c shows the comparison of the percentage concentration of supercoiled forms in samples of cisplatin-DNA complexes incubated at 25 °C and 37 °C for 24 hours.
Again, the analyses were performed for two groups of samples: i) Pt-DNA solutions, and ii) Pt-DNA films on a Ta substrate. In the solution and film samples, the proportion of the supercoiled form of Pt-DNA is less than those for DNA alone. The molar ratio of cisplatin to DNA in the solutions was 2:1. TE buffer was added to the samples in the concentration of three organic ions per nucleotide. Predictably, in both samples the supercoiled form of DNA decreased when the incubation temperature increased. The decrease is small (4.2%) in the samples of Pt-DNA solution. In contrast, there is a large decrease in the supercoiled form of the Pt-DNA film samples (20.5%). Statistical analysis also showed that the decrease is significantly different for the Pt-DNA films with different incubation temperatures (p-value: 0.0049). According to our results, the incubation temperature during preparation of the Pt-DNA solution is a substantial factor in determining the composition of Pt-DNA films on Ta substrate for use in irradiation experiments. Moreover, the results suggest that a film composed of cisplatin-DNA complexes with a high proportion of intact DNA molecules (supercoiled form) on a Ta substrate can be obtained when DNA platination occurs at 25 °C.

**Kinetics of binding Pt-Compounds to DNA.** Following platination at 25 °C, DNA has much less damage during the process of deposition onto- and recovery from the Ta substrate. However, the DNA platination reaction proceeds with a slower rate. Increasing the concentration of the Pt-compounds can compensate for this lower rate. Figure 2 shows the ratios of bound Pt-compound to DNA for different incubation times at 25 °C when the initial concentration ratios of Pt-compounds to DNA in solution are 200:1, 40:1, and 20:1. The solution consists of plasmid DNA, cisplatin or carboplatin, and tris with the ratio of 1:1 nucleotide. This amount of tris was considered as the minimum amount of buffer which can preserve the stability of DNA during the preparation process. It is clearly seen that the binding kinetics of cisplatin and carboplatin to DNA are similar and exhibit exponential fit. These curves generally reach saturation prior to 8 hours and show a linear behavior prior to 2 hours. For the initial concentration ratio of 200 cisplatin
Figure 2 Kinetics of binding of Pt-compounds to plasmid DNA. The Pt-compounds are: (a) cisplatin with the initial ratios in the solution of 20:1, (b) 200:1, and (c) carboplatin with the initial ratios of 40:1 and (d) 200:1. The curves show the quantity of bound Pt-compounds per DNA molecule at different incubation times at 25 °C. Data in a–d are means from three measurements; error bars show standard deviations. The continuous black lines are exponential fits to the data.

molecules per DNA, it is possible to have Pt-DNA samples with the ratios of bound cisplatin to DNA from 16:1 to 37:1 in 40 minutes to 120 minutes incubation time, respectively. For the same incubation times, the ratios are 2:1 and 3:1 when the initial ratio of cisplatin to DNA decreases an order of magnitude (20:1). The results demonstrate
that various ratios of bound cisplatin or carboplatin to DNA can be obtained in the incubation times of less than 2 hours by increasing the initial concentration of the Pt-compounds. Since the kinetics curves obey a linear fit for these incubation times, it is possible to simply extrapolate a variety of Pt-DNA ratios from this part of the curves.

Since Pt-compounds can react with most buffers [42], their concentration is also a relevant parameter in the DNA platination process (i.e. buffers compete with DNA for binding Pt-compounds). Tris is widely used as a buffer, especially for solutions of nucleic acids. It also reacts with Pt-compounds to produce $\text{cis-}[\text{Pt(NH}_3\text{)}_2(\text{N-Tris})(\text{OH})]^+$ and $\text{cis-}[\text{Pt(NH}_3\text{)}_2(\text{N,O-TrisH}_2)]^+$ [43]. The bar graphs in Figure 3 show a comparison of bound Pt-compounds to DNA ratios for three different incubation times at 25 °C for two different solutions: i) A mixture of DNA, cisplatin, and ddH$_2$O, and ii) a mixture of DNA, cisplatin, ddH$_2$O, and tris with the concentration ratio of 1:1 nucleotide. The initial

![Figure 3 Impact of tris on the reaction of DNA platination. Pt-DNA ratios in the cisplatin-DNA solutions incubated during 45, 90, and 180 minutes at 25 °C are compared in the presence and absence of tris. Data are means from three measurements; error bars show standard deviations.](image-url)
concentration ratio of cisplatin to the DNA was 20:1 in the solutions. The results demonstrate that the ratio of bound cisplatin to the DNA is more than double when the platination reaction occurs in a ddH₂O solution without tris compounds.

**Effects of Incubation Time on DNA and Pt-DNA Films.** The bar graphs in Figure 4 show a comparison of the percentage of supercoiled DNA and Pt-DNA samples that were incubated at 25 °C for 2, 4, and 8 hours. The analyses were performed for samples that had been recovered i) from solution, immediately after incubation (Fig. 4a), and ii) from films deposited on Ta (Fig. 4b). The Pt-DNA samples were prepared with either cisplatin or carboplatin. The initial concentration ratio of the Pt-compounds to DNA was 200:1 and that of the TE buffer was three organic ions per nucleotide. As seen from Figure 4, more than 90 percent of the DNA, in samples incubated for 2 hours, is in the supercoiled form. The proportion of supercoiled form decreases when the samples are incubated for 4 hours or more. The decrease is statistically significant in all samples except for the pure DNA solution sample. As might be expected, the decrease is greater in Pt-DNA films than in DNA samples. Since the recovery efficiency of Pt-DNA films from Ta substrate is similar to those of DNA, such a decrease in the supercoiled form indicates that Pt-compounds make plasmid DNA sensitive to any further manipulation. However, it is possible to prepare Pt-DNA films with a high proportion of supercoiled DNA at various ratios of bound Pt to DNA, by mixing DNA with high concentrations of Pt-compound solution and restricting the length of the incubation to less than 2 hours, as long as the incubation temperature does not exceed 25 °C.

**Effects of Bound Pt to DNA on Pt-DNA Samples Analysis.** The distortion of the DNA structure resulting from the formation of Pt-DNA cross-links must be considered in quantification methods such as electrophoresis. Figure 5a shows the migration of different forms of cisplatin-DNA in the electrophoresis gel. The mobility of the nicked circular, concatemeric, and supercoiled bands is changed with increasing numbers of bound Pt molecules per nucleotide (Rₚ). The change is due to distortion of the different forms of DNA by cisplatin since Pt-DNA crosslinks are known to cause conformational changes in DNA including shortening (bending) and unwinding [44, 45]. The distortion
Figure 4 Comparison of the percentages of supercoiled forms in the samples of DNA, cisplatin-DNA, and carboplatin-DNA (a) in solution, and (b) on tantalum substrate, after incubation for 2, 4, and 8 hours at 25 °C. Data are means from three measurements; error bars show standard deviations.

* indicates p-value > 0.05
** indicates p-value < 0.05
becomes greater as a function of the quantity of bound Pt molecules. Figure 5 shows the dependence of the mobility of the supercoiled, nicked circular, and concatemeric forms of cisplatin-DNA samples as a function of the ratio $R_b$ in a 1% agarose gel. The mobility of each form of Pt-DNA is normalized to the same form of an unmodified DNA sample (Fig. 5b). As seen from Figure 5b, the migration of the nicked circular and supercoiled configurations generally increases with rising in $R_b$. However, the mobility of the nicked circular form increases with a faster rate than that of the supercoiled form. Mobility of the concatemeric configuration decreases with rising in $R_b$ up to 0.009 and then increases for higher $R_b$.

Since the number of Pt molecules per plasmid probably represents a Poisson distribution for each Pt-DNA ratio, this would be expected to reduce the resolution of the agarose gels by increasing the dispersion within each band (i.e. the band width). The linear plasmid band lies between the nicked circular and concatemeric bands; thus an increase in band width could hinder precise quantification of the linear band which usually is weaker than the others. Furthermore, the nicked circular and concatemeric bands merge owing to increased band width and form one band at $R_b = 0.022$. Our results show that the mobility changes are substantial for the $R_b$ greater than 0.005.

CONCLUSION

Thin films of Platinum-DNA adducts can be considered as useful models in irradiation experiments to study the molecular mechanisms of radiosensitization which underlies concomitant chemoradiation therapy. We have investigated the optimum experimental conditions to prepare dry thin films of Pt-compounds bound to plasmid DNA on a Ta substrate. Incubation conditions in DNA platination reactions have substantial effects on the stability of Pt-DNA, particularly in the thin film samples preparation. In most of the in-vitro experiments, reaction of Pt-compounds with DNA solutions have been performed at 37 °C for incubation times varying from 24 to 48 hours. However, our results show that these conditions can induce damage to the DNA and highly sensitize them to manipulations required to form thin films and recover DNA from the Ta substrate. The concentration of intact DNA increases significantly in the film samples when the
Figure 5 Mobility of Cispatin-DNA molecules in agarose gel. (a) Migration of the different configurations of cisplatin-DNA molecules separated by electrophoresis. Lane 1 is for a DNA sample and lanes 2-5 are for cisplatin-DNA samples with the number of bound cisplatin molecules per nucleotide, R_b, of 0.0057, 0.008, 0.0091, and 0.0219, respectively. (b) Normalized mobility of the nicked circular, supercoiled, and concatemeric forms of Pt-DNA samples at different R_b in gel electrophoresis.
incubation temperature during reaction with the Pt is reduced to 25 °C and the time of incubation is 2 hours. By increasing the concentration of the Pt-compounds, it is possible to compensate for the reduced reaction rate at lower temperature and shorter time. High levels of plasmid platination however affect the quantification of Pt-DNA samples in agarose gel electrophoresis, because Pt-DNA adducts distort the conformation of DNA molecules. Therefore, the optimum condition is obtained from an equilibrium between temperature, time, and Pt-compounds concentration during the DNA platination reaction.

By recording the kinetics of binding Pt-compounds to DNA, it is possible to extrapolate different Pt-DNA ratios from the kinetics curves. We have found that the proportion of supercoiled DNA is more than 90% in the Pt-DNA film when the DNA platination reaction is performed at 25 °C for less than 2 hours in solutions containing the Pt-compounds with quantities of less than $3 \times 10^{-2}$ Pt molecules per nucleotide and the minimum concentration of Tris buffer (one tris compound per nucleotide). Under these conditions, agarose gel electrophoresis is an accurate method for quantification of DNA damage. We have also determined that the maximum number of bound Pt-compound per nucleotide is about $5 \times 10^3$ under the optimum conditions. This ratio is an order of magnitude higher than those found in biological studies and clinical applications [46]. These high ratios, however, are useful for in vitro mechanistic studies in which substantial quantities of product are required. Hence, we have found that by adjusting the initial concentration of Pt-compounds in solution, Pt-DNA films having a known controlled ratio of platinum chemotherapeutic agents to DNA can be obtained while maintaining DNA integrity.

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II.2. Second Article: Absolute Cross Section for Low Energy Electron Damage to Condensed Macromolecules: A Case Study of DNA

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Absolute Cross Section for Low Energy Electron Damage to Condensed Macromolecules: A Case Study of DNA

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ABSTRACT
Cross sections (CS) for the interaction of low energy electrons (LEE) with condensed macromolecules are essential parameters to accurately model radiation induced molecular decomposition and chemical synthesis. Electron-irradiation of dry nanometre-scale macromolecular solid films has often been employed to measure CS and other quantitative parameters for LEE interactions. Since such films have thicknesses comparable with electron thermalization distances, energy deposition varies throughout the film. Moreover, charge accumulation occurring inside the films, shields a proportion of macromolecules from electron irradiation. Such effects complicate the quantitative comparison of the CS obtained in films of different thickness, and limit the applicability of such measurements. Here, we develop a simple mathematical model, termed molecular survival model that employs a CS for a particular damage process together with an attenuation length related to the total CS, to investigate how a measured CS might be expected to vary with experimental conditions. As a case study, we measure the absolute CS for the formation of DNA strand breaks (SB) by electron irradiation at 10 and 100 eV, of lyophilised plasmid DNA films with thicknesses between 10 to 30 nm. The measurements are shown to depend strongly on the thickness and charging condition of the nanometre-scale films. Such behaviours are in accord with the model, and support its validity. Via this analysis, the obtained CS for SB damage is nearly independent of film thickness and charging effects. In principle, this model can be adapted to provide absolute CS for electron-induced damage/reaction occurring in other molecular solids across a wider range of experimental conditions.

INTRODUCTION
Electron interactions leading to molecular decomposition and synthesis within condensed matter are of relevance to problem in diverse fields such as biophysics [1-4], astrophysics and chemistry [5-7], nanotechnology [8-10], material and environmental sciences [11-14]. Both analytical and Monte-Carlo simulation methods are usually employed to quantitatively model the complex sequences of electron induced processes occurring in condensed media. Such simulations require cross section (CS) values as input data to describe all the collisions made by the primary and all secondary electrons with the
molecules of the solid or liquid under consideration [15,16]. Thus, considerable experimental and theoretical efforts have been directed to determine elastic and inelastic electron scattering cross sections (CS) for a wide range of molecules encountered in condensed media. Much of the available experimental data derives from gas-phase electron-molecule collision measurements that are free from multiple scattering effects and for which the initial and final states of the electron-target system are well characterised. However, there exist two main issues related to use of gas-phase data for condensed-phase simulations: (1) macromolecules such as polymers are not easily vaporised without molecular decomposition for gas-phase measurements [17], (2) electron-molecule scattering CS can change upon condensation due to interactions among the molecules, molecular ordering and band structure effects [18-20].

At the theoretical level, models have been developed to determine scattering CS in the condensed phase [21-23]. For moderate- and high-energy electrons, the latter provide relatively accurate values for the CS [24, 25], since the interactions of these electrons with the condensed matter arise through individual localized processes occurring at the atomic level and separated by mean free paths (MFP) much larger than the atomic dimension. In fact, such conditions are similar to those of gas-phase measurements. In contrast, at low-energy (0-100 eV) electrons have wavelengths comparable to the distance between the target molecules, and hence they interact with the condensed medium through delocalized processes predominantly including static and correlation interactions with neighbouring molecules, excitation transfer and coherent scattering [26-29]. Even though theoretical models have tried to approximate these processes and then transfer the CS data obtained from gas-phased measurements to the condensed-phase conditions, such calculated CS differ substantially from the available experimental data [29-32].

Several experimental techniques have been developed to measure various CS and MFP for LEE interactions within condensed matter [33]. In these techniques, molecules or compounds are deposited on a metal substrate by vapour condensation, sublimation, molecular self-assembly (MSA) and freeze drying (lyophilisation). A flux of LEE, provided by an external electron source under ultrahigh vacuum conditions or generated by x-ray absorption in the underlying metal substrate, is made to pass through the molecular solid film. Following LEE exposure or irradiation of the film, the analysis of the products is
performed via two general strategies. The first consists in the measurement of the energy, intensity and direction of the backscattered and/or transmitted electrons by the techniques of low energy electron transmission (LEET), electron energy loss (EEL) and low energy photoelectron transmission (LEPET) spectroscopies [34,35]. The second is the quantification of the molecular alterations such as decomposition or dimerization of the irradiated film by methods of x-ray photoelectron spectroscopy (XPS), electron simulated desorption (ESD), liquid chromatography, mass spectrometry and gel electrophoresis [35,36]. While these techniques provide valuable information on LEE interactions with condensed matter, most of the measured CS are effective, as they are dependent on the experimental conditions. Therefore, there is an essential need to improve both theoretical and experimental methods for obtaining scattering CS for LEE in condensed matter.

One approach to determine absolute CS of LEE interaction with condensed molecules is to perform a two-stream multiple-scattering analysis of the backscattered electron energy distribution measured by electron-energy-loss (EEL) spectroscopy [29,30,37,38]. As a first step, entire EEL spectra along with the transmitted currents are measured at different molecular coverage for a fixed incident energy. From the linear relationship found between the energy integrated EEL spectra and the corresponding transmitted currents, a differential incident electron current is established. The intensity scale of the EEL spectra are then normalised to this current so as the area under an elastic and inelastic feature can be expressed in terms of an absolute reflectivity. When the film thickness is smaller than the MFP of the incident electron (i.e., single collision regime), the same area with the knowledge of the molecular coverage gives immediately the absolute CS for electrons backscattered over the whole half-angular space [29, 38]. In the case of isotropic scattering, the latter amounts to half of the integral CS. When the film thickness is comparable to or larger than the MFP of the incident electron (i.e., multiple collision regime), the energy dependent elastic electron reflectivity (i.e., electron scattered elastically) measured as a function of the film thickness leads to the absolute value of the energy dependent total CS (i.e., inverse MFP) [30, 37]. The latter is then used to normalise the relative elastic and inelastic integral CS, which are obtained relatively to the total CS from a detailed two-stream multiple-scattering analysis of the EEL spectra. Since such measurements of the absolute CS are limited in practice to films whose thicknesses may
range from 1 to about 3 times of MFP of the incident electron, they cannot be performed with aggregate of macromolecules at film thicknesses already much larger than the expected electron MFP.

Nanoscale thin solid films of macromolecules are usually prepared via MSA and lyophilisation techniques [39-41]. Although both techniques provide thin macromolecular films suitable for studying LEE interactions, each method has some weakness that may affect the results of LEE-macromolecule scattering experiments. While films formed by MSA techniques have a superior uniformity to those prepared by lyophilisation, they invariably contain some additional molecular species or molecular modifications which are necessary to bind the macromolecules together or onto the substrate. Such additions or modifications may affect the macromolecule conformation and modulate the damage induced by LEE [42]. In contrast, by lyophilisation, it is possible to prepare pure films of macromolecules or in controlled mixture with other molecules such as potential radiosensitizers [43,44]. However, the main disadvantage is the ill-defined morphology of the films [45,46], since the films have irregular thickness and uniformity due to the formation of macromolecular aggregates. Nevertheless, lyophilisation has been a useful technique for studying the effect of LEE on condensed matter [47,48].

So far, the CS of LEE-induced damage to a supercoiled plasmid DNA have been estimated merely from measuring the percentage of the loss of SC DNA as a function of irradiation time $t$ (i.e., exposure-response curve) and quantifying configurational changes of the plasmid [49]. The supercoiled DNA changes to circular and linear forms following single and double strand breaks (SB), respectively. However, with a double helix diameter of about 2-3 nm, a SC DNA is already comparable to or larger than the expected MFP of LEE. Therefore, such large molecules in the topmost part of a heterogeneous film hide those located behind them from the incoming electrons. This phenomenon is akin to an attenuation length (AL) for the incident LEE inside the film and thus limits the LEE-induced damage to a fraction of the film. Besides, most of the LEEs suffer energy loss inside the DNA molecules to end up into intermolecular traps, or via dissociative electron attachment (DEA), stabilize as atomic or molecular anions leading to film charging [50-54]. Both AL and film charging effects can lead to considerable error in the CS values for the formation of DNA damage if not accounted for in the data analysis.
The present paper introduces a molecular survival model adapted to the phenomenology observed in LEE-irradiation experiments with lyophilised plasmid DNA films. The effect of AL of LEE on the calculated exposure-response curve is investigated as a function of the film thickness. The exposure-response curve for a given AL of LEE and film thickness is simulated in absence and gradually presence of film charging. An absolute CS value of LEE-induced damage that account for the AL and film charging effects are obtained from experiments with lyophilised plasmid DNA films irradiated with 10 and 100-eV electrons. Finally, using the AL values found in the present work, a “penetration factor” is introduced to allow comparison with previous CS measurements.

EXPERIMENTAL METHOD

The experimental details of sample preparation, irradiation, and post-irradiation analysis techniques employed in the present studies have been reported in detail elsewhere [55,56]. Here, we provide only brief description of the most pertinent elements.

Plasmid DNA [pGEM-3Zf(-), 3197 base pairs, ca. 1,968,966 amu per plasmid] was extracted from Esherichia coli JM109 and purified with a HiSpeed plasmid Maxi kit (QIAGEN). The purified plasmid DNA consisted of 97% supercoiled, 2% concatemeric, and 1% nicked circular forms. The concentration of DNA and the relative quantity of proteins in the plasmid DNA solution was then calculated by measuring the ratio of ultraviolet (UV) absorption of DNA and protein at 260 nm and 280 nm, respectively, with a Synergy HT-I spectrophotometer. The ratio was 1.99 which corresponds to a purity greater than 95% [56]. The TE buffer (Tris-EDTA: 10 mM/1 mM) was separated from DNA by gel filtration with a Sephadex G-50 medium. Thus, the final solution consisted of DNA and ddH$_2$O after the filtration.

The solution of pure DNA was split in four parts and each of them further diluted in ddH$_2$O to obtain the concentrations of 30, 45, 60 and 90 ng/μl DNA. To make 5 to 15 monolayer (ML) films of plasmid DNA, 7 μl of the DNA solutions was deposited onto clean tantalum (Ta) substrates (7 x 20 mm). The latter consisted of a thin layer (450 ± 50 nm) of Ta sublimated onto either a 0.4 mm thick silicon wafer or a clean borosilicate glass. Then the deposited DNA samples were first frozen at -65°C for 10 minutes in a glove box and then dried under a pressure of about 6 mTorr by a hydrocarbon-free turbomolecular
RESULTS – Second Article

pump for 2 hours to form solid films. The DNA films were circular in shape with an average radius $r = 2$ mm ($\pm$ 5%). Using the known density $\rho = 1.7$ g/cm$^3$ ($\pm$ 5%) for the plasmid DNA extracted from *E. coli* (i.e., the buoyant density of DNA) and the masses $m$ of 210, 315, 420, 630 ng ($\pm$ 10%) of DNA contained in the 7 $\mu$l drops deposited on Ta prior to lyophilisation, their average thicknesses $h = m/2\pi r^2 \rho$ were calculated to be 10, 15, 20 and 30 nm with an uncertainty of $\pm$ 13%.

After preparation, the DNA films were placed on sample holders inside a UHV chamber equipped with an electron irradiator. The latter consists of an electron gun producing a beam adjustable in energy between 5 and 1000 eV. The spot size of the beam can be varied between 2 and 50 mm at working distances of 10 and 50 mm. In the present experiment, it was set to irradiate an area of about 0.9 cm$^2$ which was 7 times larger than the DNA sample. The chamber was evacuated for 24 hours by a hydrocarbon-free turbomolecular pump to a pressure of $5 \times 10^{-9}$ Torr at room temperature.

After stabilization of the electron beam current at 2 nA ($\pm$ 5%) [56], corresponding to the current density of $9.95 \times 10^{10}$ electron s$^{-1}$ cm$^{-2}$, the DNA films were individually irradiated with electrons of either 10 or 100 eV for periods between 5 and 90 seconds. While bombarding a sample, the others were shielded from stray electrons by applying a repulsive potential of 9 V with respect to the cathode of the electron gun. One of the samples in the UHV chamber was never irradiated with electrons to serve as a control.

After irradiation, the samples were removed from the chamber and immediately dissolved in 10 $\mu$l of TE buffer at pH 8.0. A comparison of the amount of recovered DNA with the original solution used for DNA deposition showed that nearly 98% of the deposited DNA was recovered from the substrate. The separation of the different structural forms of DNA, such as supercoiled, nicked circular, linear, etc., in the samples was performed by agarose gel electrophoresis. The DNA samples and the agarose gels were stained with SYBR Green I in the concentration of 100X and 10,000X, respectively. The samples were passed on 1% agarose gel in 1X TAE buffer at 100 volts for 7 minutes followed by 75 volts for 68 minutes (5 V cm$^{-1}$). The gels were then scanned by Typhoon-Trio laser scanner (from GE Healthcare) adjusted for the blue fluorescent mode at an excitation wavelength of 488 nm and filter type 520 nm-bandpass (520 BP 40) in the normal sensitivity mode. The amount of each structural form of the DNA was analyzed by
ImageQuant (Molecular Dynamics) software. To achieve a better accuracy, the binding efficiencies of SYBR Green I for the same amount (75 ng) of supercoiled and linear DNA was measured to established a correction factor. This factor which arises from the weaker binding of SYBR Green I to supercoiled DNA than to the nicked circular and linear forms, was 1.2 and applied to the quantification of the different structural forms of plasmid DNA.

**MOLECULAR SURVIVAL MODEL**

Molecular damage such as DNA SB results from the inelastic interactions of incident electrons with macromolecule subunits and may involve ionization, excitation, and the formation of negative ions leading to the rupture of bonds within and between the constituents [55,56,58,59]. Elastic and inelastic collisions contribute to the spread of an incident electron beam within a film. Surface analysis of multilayer films by x-ray photoelectron spectroscopy shows that the decrease in electron intensity depends exponentially on the thickness of the films [60-63]. Similarly, for a monoenergetic incident electron beam with a uniform surface current density $J_0$ impinging on a molecular film of thickness $h$, the surface current density $J(x)$ at a depth $x$ inside the film is given simply by:

$$J(x) = J_0 e^{-\frac{x}{\lambda}} , \quad (1)$$

where $\lambda$ is defined as the AL.

Let $q(x,t)$ be defined as the relative proportion of intact molecules (e.g. SC DNA) within an infinitesimally thin slab between $x$ and $x + dx$ after an irradiation time $t$. The value of $q(x,t)$ is unity when there is no molecular damage at $t = 0$ and decreases toward zero with $t$. Integrating $q(x,t)$ over the whole film thickness between 0 and $h$ gives the total percentage of intact molecules in the film at a given $t$:

$$P(t) = P_0 \frac{1}{h} \int_0^h q(x,t)dx , \quad (2)$$

where $P_0$ is the percentage of intact molecules in the non-irradiated film. The function $q(x,t)$ is solution of

$$dq(x,t) = -\alpha J(x)q(x,t)dt , \quad (3)$$
where $\sigma$ is the CS to damage a molecule by LEE impact (e.g., loss of SC DNA). By substituting the right-hand side of Eq. (1) into Eq. (3) and using the initial condition $q(x,0) = 1$, Eq. (3) is readily solved as

$$q(x,t) = e^{-\alpha \frac{x}{\lambda}}. \quad (4)$$

By substituting the above expression for $q(x,t)$ into Eq. (2), $P(t)$ becomes:

$$P(t) = P_0 \frac{1}{h} \int_0^h e^{-\alpha \frac{x}{\lambda}} \, dx. \quad (5)$$

It is also possible to algebraically solve the integral with the following change of variable:

$$z = e^{-\frac{x}{\lambda}} \quad \text{and} \quad dz = -\frac{z}{\lambda} \, dx$$

$$P(t) = P_0 \frac{-\lambda}{h} \int_0^h \left( e^{-\alpha \frac{z}{\lambda}} \right) \, dz. \quad (6)$$

Expanding the numerator of the integral in series around $t = 0$ and integrating each term separately, the solution of the Eq. (6) can be written as:

$$P(t) = P_0 \left[ 1 - f_1 \frac{(\alpha J_0)}{!} t + f_2 \frac{(\alpha J_0)^2}{2!} t^2 - f_3 \frac{(\alpha J_0)^3}{3!} t^3 + \ldots \right], \quad (7)$$

where

$$f_n = \left( \frac{\lambda}{nh} \right) \left( 1 - e^{-\frac{nh}{\lambda}} \right),$$

with $n = 1, 2, 3, \ldots$. Eq. (7) gives the total percentage of intact molecules in the film following an irradiation time $t$. For a sufficiently small $t$, the expression for $P(t)$ reduces to the first two terms

$$P(t) = P_0 - P_0 f_1 \alpha J_0 t = P_0 - P_0 \alpha J_0 \left( \frac{\lambda}{h} \right) \left( 1 - e^{-\frac{h}{\lambda}} \right) t, \quad (8)$$
with $P(t)$ varying linearly with $t$ along with the proportionality constant depending on $P_0$, $\sigma$, $J_0$, $\lambda$ and $h$. It should be noted that $\lambda$ is connected with the total CS and density of the material and therefore depends implicitly on $\sigma$. However, with the present model $\lambda$ and $\sigma$ can be determined separately.

The simulated exposure-response curves derived from Eq. (5) for solid DNA films of 10, 20 and 40 nm for a fixed $\lambda$ of 12 nm and $\sigma$ of $5 \times 10^{14}$ cm$^2$ are plotted in Fig. 1. The dependence of the initial slopes on $h$ indicates that the rate of SC loss decreases with the film thickness. From the calculated curves, the fraction of DNA in the SC forms is expected to decrease quasi exponentially to zero with increasing $t$. However, this behavior differs from the experiments in which the exposure-response curves reach finite levels [55,56]. These saturation effects are the result of charge accumulation within the irradiated films (i.e., film charging).

Film charging is an inevitable consequence of the irradiation of a thick molecular solid film with LEEs. Such films can charge negatively or positively depending on the relative proportion of secondary electron emission, ionization, electron thermalisation and DEA processes. For electron irradiation at energy less than that required for ionization, electron trapping leads to the accumulation of negative charges in the films. Previous studies have shown that a submonolayer of molecules deposited onto the surface of a dielectric film deposited onto a metallic substrate, can trap electrons and thus generate a negative potential acting as a barrier for the incoming electrons [50,64-67]. The magnitude of this potential barrier depends on the accumulated charge density and its distance from the metallic substrate (i.e., the thickness of the dielectric layer $L$). For small $L$, the charges are close to the substrate and therefore only a small potential barrier is generated whereas for large $L$ the charge is far from the substrate resulting in a much larger potential. When considering molecular solids, such as lyophilised DNA, and the condition $h > \lambda$, one expects an accumulation of negative charges in the topmost part of the film far from the metallic substrate thus giving rise to a large repulsive potential. Since the thickness of the dielectric layer $L$ increases with film thickness $h$, the magnitude of the repulsive potential increases with $h$. 
Lyophilised films of macromolecules such as DNA have thickness irregularities due to aggregation. When the incident electrons get trapped within relatively thick aggregates, they give rise to a large repulsive potential even if the rest of the film bears very few charges. This repulsive potential, which increases with the electron irradiation time, subsequently repels the incoming electrons. When considering the random accumulation of charges over a film surface, the number of sites accessible to the incident electrons (i.e., non-charged sites) should decrease exponentially upon electron irradiation time. Consequently, the electron density \( J_0 \) impinging on the film can be considered to decrease exponentially with the irradiation time.

Under these conditions, the charging effect can be simply accounted for by modifying Eq. (1) as follows:
\[ J(x,t) = J_0 e^{\frac{-x}{\lambda} e^{\frac{-t}{\tau}}} \],

where \( \tau \) is a charging time constant that characterises the decrease of \( J_0 \) with the irradiation time \( t \). When \( \tau \to \infty \) or if \( t >> \tau \), as might be the case with thinnest films, Eq. (9) reduces to Eq. (1) in absence of charging condition. So in presence of charging, \( q(x,t) \) and \( P(t) \) are rewritten as:

\[ q(x,t) = e^{-\alpha_0 \sigma (1-e^{-\tau}) e^{\frac{-x}{\lambda}}} \],

and

\[ P(t) = P_0 \int_0^\infty \frac{r^h}{h} e^{-\alpha_0 \sigma (1-e^{-\tau}) e^{\frac{-x}{\lambda}}} dx \].

In Fig. 2(a), Eq. (10) is used to simulate how \( q(x,t) \) varies within a film in absence of charging (i.e. \( \tau \to \infty \)) and Fig. 2(b) in presence of charging. Each solid line in the figure corresponds to \( q(x,t) \) after a given irradiation time. In the case of zero charging and for \( x \ll \lambda \), \( q(x,t) \) decreases rapidly with irradiation time. Deeper within the film, as \( x > \lambda \), a relatively smaller fraction of the target molecule (SC DNA) is lost upon electron irradiation. When the charging effect is included in the calculation (Fig. 2b), the number of accessible sites is further reduced, and the molecular damage is restricted to a much smaller fraction of the film, close to \( x = 0 \).

Fig. 3 shows how \( P(t) \) calculated from Eq. (11) varies as a function of the irradiation time \( t \) for a 40 nm thick DNA film given different charging time constant \( \tau \), along with the values of \( P_0 \), \( \sigma \), \( J_0 \) and \( \lambda \) being the same as Fig. 1. As might be anticipated, the smaller value of \( \tau \), the larger the asymptotic value of \( P(t) \) at long \( t \) and the shorter the time required for \( P(t) \) to reach this saturation value. In other words, the quicker the film charges, the smaller the fraction of the film that is irradiated and the smaller the loss of SC DNA. On the other hand, the slopes of the curves for different \( \tau \) at \( t = 0 \) are found to be the same. Therefore, the rate of decrease of the initial concentration of the target molecules is in principle independent of film charging. In other words, for sufficiently short irradiation
Fig. 2. Simulation of the normalized number of SC DNA in various depths of a DNA film at different irradiation times (t) in absence of charging (a) and in presence of charging (b).
time, the charging should have the least effect on the slope of the exposure-response curve and still be given by [c.f., Eq. (8)],

\[ P'(0) = -P_0 \alpha J_0 \left( \frac{\lambda}{h} \right) \left( 1 - e^{-\frac{h}{\lambda}} \right). \]  

\[ (12) \]

RESULTS and DISCUSSION

AL and CS for DNA strand breaks at 10 and 100 eV

Fig. 4 presents the measured percentage loss of SC DNA as a function of irradiation time \( t \) (i.e., exposure-response curves) for 10 – 30 nm thick lyophilized pure DNA films exposed to monoenergetic electrons of 10 and 100 eV. As expected, the proportion of DNA in the SC form decreases with \( t \) as it primarily transformed into nicked circular and linear forms via the induction of SSB and DSB, respectively. Similar to the simulations in Figs. 1 and 3,
Fig. 4: Exposure response curve for lyophilized plasmid DNA films of 10, 15 and 20 nm average thickness, irradiated with 10-eV electrons (a) and 10, 20 and 30 nm average thickness, irradiated with 100-eV electrons (b). The dash-point lines are guides for eye. Each data point corresponds to the mean value of three samples with the relevant standard deviation.
the initial portions of the measured exposure-response curves at short $t$ exhibit a near linear behaviour whose slope becomes less steep as $h$ is increased. The values of these slopes obtained using only points up to 10 s are, respectively, 0.23 $\pm$ 0.03, 0.2 $\pm$ 0.02 and 0.15 $\pm$ 0.02 s$^{-1}$ for the 10, 15 and 20 nm films exposed to 10-eV electrons, and 0.47 $\pm$ 0.1, 0.37 $\pm$ 0.09 and 0.27 $\pm$ 0.05 for the 10, 20 and 30 nm films exposed to 100-eV electrons. At large $t$, each curve reaches a saturated level due to the effect of a finite AL and film charging effect. These results are in good agreement with the characteristics expected for exposure-response curves by our model (Fig. 3). These correspondences provide assurance that CS and AL values can be deduced from the experimental data by employing the proposed molecular survival model.

Using Eq. (12), $\lambda$ can be determined from the ratio $R_{1,2}$ of the initial slopes $P_1'(0)$ and $P_2'(0)$ of the exposure-response curves between two different thicknesses $h_1$ and $h_2$:

$$R_{1,2}(\lambda) = \frac{P_1'(0)}{P_2'(0)} = \frac{-P_{01}\alpha J_0\left(\frac{\lambda}{h_1}\right)\left(1 - e^{-\frac{h_1}{\lambda}}\right)}{-P_{02}\alpha J_0\left(\frac{\lambda}{h_2}\right)\left(1 - e^{-\frac{h_2}{\lambda}}\right)} = -\frac{P_{01}h_2\left(1 - e^{-\frac{h_2}{\lambda}}\right)}{-P_{02}h_1\left(1 - e^{-\frac{h_1}{\lambda}}\right)}.$$  (13)

Since $P_1'(0)$, $P_2'(0)$, $P_{01}$, $P_{02}$, and $h_1$ and $h_2$ are the known experimental parameters, it is possible to obtain a statistical average value for $\lambda$ by repeating the analysis for all possible pairs among the three thicknesses studied in our experiment. Once $\lambda$ and its uncertainty are determined, then $\sigma$ is readily obtained from Eq. (12).

Table 1 presents the ratios of the initial slopes obtained between all possible pairs $h_1$ and $h_2$ among the 10, 15 and 20 nm films at 10 eV and 10, 20 and 30 nm films at 100 eV along with the corresponding deduced values of $\lambda$ and their statistical uncertainties from Eq. (13). We observe that at both incident electron energies, the ratios of initial slope yield progressively smaller $\lambda$ values as the film thickness increases. By comparing the effect of a finite $\lambda$ on the loss of SC DNA with electron exposure in Fig. 1 and Fig. 3 (i.e., the effect of film charging, $\tau$), we further note that within a given range of exposure, a decrease in $\lambda$ and increase in charging rate (i.e., decrease in $\tau$) produce a similar effect; that is, they both act to reduce the size of any loss of SC DNA. It is therefore suggested that the trend of
Table 1. Values for the ratios $R_{i,j}(0)$ of the initial slope of the exposure-response curves between all possible thickness pairs $h_j$ (nm) and $h_2$ (nm) among the 10, 15 and 20 nm films at 10 eV, as well as the 10, 20 and 30 nm films at 100 eV. Corresponding values of the AL in the plasmid DNA, $\lambda$ (nm), deduced from Eq. (13) along with the calculated average. SE is standard error.

<table>
<thead>
<tr>
<th></th>
<th>$h_1$</th>
<th>$h_2$</th>
<th>$R_{i,j}(0)$</th>
<th>$\lambda$</th>
<th></th>
<th>$h_1$</th>
<th>$h_2$</th>
<th>$R_{i,j}(0)$</th>
<th>$\lambda$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 eV</td>
<td>10</td>
<td>15</td>
<td>1.15 ± 0.2</td>
<td>16.0 ± 3.7</td>
<td>10</td>
<td>20</td>
<td>1.27 ± 0.4</td>
<td>18.1 ± 6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>1.47 ± 0.3</td>
<td>9.8 ± 3.7</td>
<td></td>
<td>10</td>
<td>30</td>
<td>1.74 ± 0.5</td>
<td>14.0 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>20</td>
<td>1.28 ± 0.2</td>
<td>5.5 ± 3.6</td>
<td>20</td>
<td>30</td>
<td>1.37 ± 0.4</td>
<td>9.7 ± 6.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average ± SE 10.4 ± 5.4</td>
<td></td>
<td>Average ± SE 13.9 ± 5.5</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

decreasing $\lambda$ values with larger film thicknesses reflects film charging. Because of this effect, we report in Table 1 the average values of $\lambda$ at 10.4 ± 5.4 and 13.9 ± 5.5 nm for 10- and 100-eV electrons by including an additional systematic error of ± 50% and ± 30%, respectively.

Given the average $\lambda$, we present in Table 2 the values for $\sigma$ calculated from Eq. (12) for the DNA films of three different thicknesses irradiated with 10- and 100-eV electrons. The resulting uncertainty for each value depends on that of the initial slope $P'(0)$ at the range of ± 10 to 13.3% and ± 17 to 24% for 10- and 100-eV electrons, respectively, the percentage of the SC DNA in the non-irradiated samples $P_0$ at ± 1%, the film thickness $h$ at ± 13%, and the average $\lambda$ at ± 52% and 39% for 10 and 100 eV, respectively. The average values $\sigma$ for three films are $(3.8 ± 1.2) \times 10^{-14}$ and $(7.2 ± 2.1) \times 10^{-14}$ cm$^2$ for 10- and 100-eV electrons. Their uncertainties are obtained based on the weighted mean and the error in the mean [68]. In Fig. 5, the initial slopes based on the average values for $\sigma$ and $\lambda$ at 10- and 100-eV electrons are compared and fitted to the experimental data.
Table 2. Cross Section $\sigma \left(10^{14} \text{ cm}^2\right)$ to induce a SB in plasmid DNA by 10- and 100-eV electron impact on different film thicknesses $h$ (nm). SE is standard error.

<table>
<thead>
<tr>
<th>$h$ (nm)</th>
<th>$10 \text{ eV}$</th>
<th>$100 \text{ eV}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.7 ± 2.1</td>
<td>7.1 ± 3.8</td>
</tr>
<tr>
<td>15</td>
<td>3.9 ± 2.2</td>
<td>7.5 ± 3.8</td>
</tr>
<tr>
<td>20</td>
<td>3.7 ± 2.0</td>
<td>7.1 ± 3.2</td>
</tr>
<tr>
<td>Average ± SE</td>
<td>3.8 ± 1.2</td>
<td>Average ± SE</td>
</tr>
</tbody>
</table>

**Correction Factor**

Based on the Eq. (8) and (12), the CS of DNA SB can be calculated from the initial slope of the exposure-response curve, when $J_0$ and $P_0$ are the known parameters. Moreover, the CS should be corrected by $f_i$ called the penetration factor when a DNA film has a thickness comparable to or larger than $\lambda$:

$$f_i = \frac{\lambda}{h} \left(1 - e^{\frac{h}{\lambda}}\right),$$  \hspace{1cm} (14)

along with

$$\sigma_{SB} = -\frac{P'(0)}{P_0 J_0 f_i}.$$

Eq. (15) thus prescribes that both the $f_i$ factor and the slope of the exposure-response curve at zero dose are essential to determine the CS of DNA SB that is independent of film thickness and charging effects.

Table 3 summarizes the CS data for DNA SB damage measured in this and several other studies by irradiation of dry, nanoscale DNA films with LEEs. The table also contains $f_i$ calculated for those experiments performed with thick films of DNA, so that the reported
Fig. 5. Calculation of the initial slope of the exposure-response curve based on the theoretical model and fitting to the measured data sets for 10-eV (a) and 100-eV (b) electrons.
CS per plasmid ($\sigma'$) are corrected to new values ($\sigma$). The relative errors in $f_i$ at about 55% and 45% for 10- and 100-eV electrons result from propagating in Eq. (14) the uncertainty in $\lambda$ and $h$ [68]. For 10-eV electrons, the present CS is similar to those recorded by Panajotovic et al. [56] and Boulanouar et al. [69], and is slightly larger relative to that measured by Dumont et al. [70]. To compare the results obtained from thick and thin films, $\sigma$ are normalized to the CS per nucleotide ($\sigma_n$) for the induction of SB. Cai et al. [61] and Dugal et al. [71] directly measured $\sigma_n$ via irradiation of a self-assembled monolayer (SAM) oligonucleotide film with LEEs. For 10-eV electrons, the present value of $\sigma_n$ is in a good agreement with those measured directly at 8-, 9- and 12-eV electrons by Cai et al and Dugal et al. These results suggest that the penetration factor $f_i$ is a crucial parameter in obtaining CS of DNA SB damage independent of the thickness of lyophilized films, particularly when the average thickness of the DNA film is comparable with the AL of incident electrons. This factor however has a limit. As film thickness increases or $\lambda$ decreases, film charging can affect substantially the initial slope of the exposure-response curves, even at short irradiation times. In our study, this effect is observed for 30 nm films irradiated with 10-eV electrons. Under this condition, Eq. (12) which is in fact obtained from first two terms of the algebraic solution of Eq. (11) is no longer accurate for calculation of AL and CS from the measured data set. For employing Eq. (12) and $f_i$ to the experimental data set, it is suggested to consider the ratio of the film thickness to AL of the incident electrons ($h/\lambda$). For 10-eV electrons, for example, the applicability of Eq. (12) is restricted to $h/\lambda \leq 2$ for the plasmid DNA films, i.e. for thicknesses up to 20 nm. Beyond the ratio, it is suggested to consider other terms of the solution of Eq. (11) by which the initial slope $P'(0)$ depends on the charging time constant $\tau$ in addition to the other parameters mentioned in Eq. (12).

Since the interaction of a LEE with a condensed molecule is affected by the neighboring molecules (e.g., through processes of target polarization and electron correlation), film morphology may affect the CS measurement. However, the similarity between the CSs of DNA SBs obtained from two different film morphologies in the present study and those measured via self-assembled film of DNA [61, 69, 71], suggests that film morphology has only a small effect on the CS, when it is corrected for different film thicknesses and charging conditions. Therefore, our model appears as an appropriate tool to
Table 3: Present and previous CS data to induce SB in DNA by LEE impact. The $f_i$ value is the penetration factor used to correct the measured CS obtained from thick films. $\sigma'$ ($10^{14}$ cm$^{-2}$) and $\sigma$ ($10^{14}$ cm$^{-2}$) present the measured CS per plasmid for loss of SC before and after applying $f_i$, respectively. $\sigma_n$ ($10^{17}$ cm$^{-2}$) stands for the CS per nucleotide to induce DNA SB, which resulted from the dividing of $\sigma$ by the number of nucleotides in the plasmid (i.e., 6394).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Film Medium</th>
<th>Film Preparation Method</th>
<th>Electron Energy (eV)</th>
<th>Film Thickness (nm)</th>
<th>$f_i$</th>
<th>$\sigma'$</th>
<th>$\sigma$</th>
<th>$\sigma_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study Plasmid DNA</td>
<td>Lyophilization</td>
<td>10</td>
<td>0.64 ± 0.35</td>
<td>2.4 ± 0.4</td>
<td>3.7 ± 2.1</td>
<td>0.58 ± 0.32</td>
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<tr>
<td></td>
<td></td>
<td>15</td>
<td>0.53 ± 0.29</td>
<td>2.1 ± 0.2</td>
<td>3.9 ± 2.2</td>
<td>0.61 ± 0.33</td>
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<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.44 ± 0.23</td>
<td>1.6 ± 0.2</td>
<td>3.7 ± 2.0</td>
<td>0.57 ± 0.31</td>
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</tr>
<tr>
<td>This study Plasmid DNA</td>
<td>Lyophilization</td>
<td>100</td>
<td>0.71 ± 0.35</td>
<td>5.04 ± 1.1</td>
<td>7.1 ± 3.8</td>
<td>1.11 ± 0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.53 ± 0.24</td>
<td>3.95 ± 0.98</td>
<td>7.5 ± 3.8</td>
<td>1.16 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panajotovic et al. 2006* [56]</td>
<td>Plasmid DNA</td>
<td>Lyophilization</td>
<td>10</td>
<td>0.4 ± 0.2</td>
<td>1.1 ± 0.5</td>
<td>2.7 ± 1.8</td>
<td>0.42 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Dumont et al. 2010 [70]</td>
<td>Plasmid DNA</td>
<td>Lyophilization</td>
<td>10</td>
<td>0.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>0.23 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Boulanouar et al. 2012 [69]</td>
<td>Plasmid DNA</td>
<td>Self-assembled</td>
<td>10</td>
<td>10, 15, 20</td>
<td>NA'</td>
<td>5.2 ± 1.3</td>
<td>NA</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Cai et al. 2006 [61]</td>
<td>Oligonucleotide</td>
<td>Self-assembled monolayer</td>
<td>8</td>
<td>&lt; 2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Dugal et al. 1999 [71]</td>
<td>Oligonucleotide</td>
<td>Self-assembled monolayer</td>
<td>9</td>
<td>&lt; 2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The corrected CS recorded by Panajotovic et al. is for formation of circular DNA which contributes to more than 95% of loss of SC.

' NA = Not applicable
measure the CS of DNA SB under condensed-phase conditions with a negligible effect from film morphology.

It has also been suggested that 10-eV electrons predominantly generate fragmentation in DNA by single events through the process of DEA via core-excited resonances [39,72], whereas 100-eV electrons may cleave DNA by multiple events through non-resonant mechanisms including ionization, excitation and neutral dissociations [73]. As a result of the multiple events, secondary species such as LEEs can be created by incident 100-eV electrons during their passage through the film. These LEEs can penetrate further into the film to induce more damage to DNA. Therefore, it is expected to have AL and CS of DNA SB at 100-eV are larger than those for 10-eV, as observed experimentally. This difference can also be seen in the measured exposure-response curves for 10- and 100-eV electrons (Fig. 4) by comparing their saturation levels, as they are lower at 100-eV than those at 10-eV. However, the CS for 100-eV electrons slightly increase, by a factor of 1.9 compared to that for 10-eV electrons. This small increase suggests that although the CS for sum of the ionization and fragmentation channels at 100-eV is reported to be larger by one or two orders of magnitude than that at 10-eV for most organic molecules [74-76], resonant and non-resonant mechanisms have relatively similar contributions to generate DNA SB at both energies. Similar comparisons were also made in the previous studies (i.e., the yields for the formation of SSB and DSB by 10- and 100-eV electrons had comparable values) [73,77,78]. Therefore, our results confirm the previous suggestion that for a given CS value, the formation of transient anion and DEA are much more efficient processes than others to induce SB in DNA.

CONCLUSION

We have developed a simple molecular survival model to obtain CS for LEE damage to a macromolecule within a nanoscale thickness solid film, which takes into account the thickness and charging of the film. It was shown that the slope of the curves at short irradiation time decreases with thickness and yields of damage reach saturation after the degradation of only a few percent of the intact molecule, owing to charge accumulation. Since the CS for molecular damage is directly calculated from such curves, particularly the initial portion of the curves at short exposure times, these effects can cause the CS to be significantly underestimated. Thus, the model allows studying the behaviour of the dose-
RESULTS – Second Article

response curves as a function of film thickness and saturation levels at high dose. It essentially evaluates the effects of film thickness and charging on the exposure-response curve for a particular damage induced by LEEs.

As a case study, we have prepared nanoscale films of pure plasmid DNA via lyophilisation technique and subsequently bombarded them with LEEs at various fluences. The exposure-response curves for induction of DNA SB was found to strongly depend on the thickness and charging of the films, in agreement with our mathematical model. Based on the latter, the effects of charge accumulation and thickness of the lyophilized films can be ignored, if the initial slope of the exposure-response curves is obtained at zero dose and corrected by a penetration factor. This factor depends on the film thickness and AL of the incident electrons inducing DNA SB. Furthermore, compatibility between the CS obtained by the model in the lyophilised films to those measured by MSA films of different types of DNA (e.g. oligonucleotide) suggests that film composition and morphology has a minor effect on the CS. Therefore, this model eliminates the major obstacles that prevent precise quantification of DNA damage and further allows the previously measured CS to be converted to a CS that is nearly independent of the film thickness and charging effects.

Considering that the measurement of the real absolute CS for the interaction of LEEs with a single DNA molecule is not currently feasible, the present CS derived herein for condensed DNA films appear to be the most precise quantities yet to describe LEE-induced damage. Moreover, the present model can be adapted to other organic and inorganic macromolecules such as synthetic polymers, nanotubes, graphene, etc. to determine an absolute CS for LEE-induced chemical processes.

ACKNOWLEDGMENTS

Financial support for this work was provided by the Canadian Institute of Health Research (CIHR).
REFERENCES:

[39] Leon Sanche, Low-energy electron interaction with DNA: Bond dissociation and formation of transient anions, radicals, and radical anions (John Wiley & Sons, New Jersey, 2009), p. 239.
II.3. Third Article: New insights into the mechanism underlying the synergistic action of ionizing radiation with platinum chemotherapeutic drugs: The role of low-energy electrons.

Authors: Mohammad Rezaee, Darel J. Hunting, Léon Sanche


Contributions: Mohammad Rezaee designed and performed research, analyzed data and wrote manuscript. Professor Leon Sanche and Professor Darel Hunting supervised the project, advised on the design and data interpretation, and edited the manuscript.
New Insights into the Mechanism Underlying the Synergistic Action of Ionizing Radiation with Platinum Chemotherapeutic Drugs: The Role of Low-Energy Electrons

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Conflict of interest: none
SUMMARY
Concurrent administration of platinum chemotherapeutic drugs (Pt-drugs) and radiation improves the treatment of several solid tumours by enhancing local therapy; however, the underlying mechanisms of the synergistic action between Pt-drugs and radiation remain the subject of active investigation. We report that Pt-drugs preferentially enhance the formation of cluster damage to DNA by ionizing radiation and that LEEs play the major role in the induction of such lesions through quantum process known as electron resonances.

ABSTRACT
Purpose: To investigate the efficiencies of platinum chemotherapeutic drugs (Pt-drugs) in the sensitization of DNA to the direct effects of ionizing radiation and to determine the role of low-energy electrons (LEEs) in this process.

Methods and Materials: Complexes of supercoiled plasmid DNA covalently bound to either cisplatin, carboplatin or oxaliplatin were prepared in different molar ratios. Solid films of DNA and DNA modified by Pt-drugs were irradiated with either 10-keV or 10-eV electrons. DNA damages were quantified by gel electrophoresis, and the yields for damage formation were obtained from exposure-response curves.

Results: The presence of an average of two Pt-adducts in 3199-bp plasmid DNA increases the probability of a double-strand break by factors of 3.1, 2.5 and 2.4 for carboplatin, cisplatin and oxaliplatin, respectively. Electrons with energies of 10-eV and 10-keV interact with Pt-adducts to preferentially enhance the formation of cluster lesions. The maximum increase in radiosensitivity per Pt-adduct is found at ratios up to $3.1 \times 10^4$ Pt-adducts per nucleotide which is equivalent to an average of two adducts per plasmid. Carboplatin and oxaliplatin show higher efficiencies than cisplatin in the radiosensitization of DNA. Since carboplatin and cisplatin give rise to identical reactive species which attach to DNA, carboplatin must be considered as a better radiosensitizers for equal number of Pt-adducts.

Conclusion: Pt-drugs preferentially enhance the formation of cluster damage to DNA by ionizing radiation and LEEs are the main species responsible for such enhancement via the formation of electron resonances.
INTRODUCTION

Concomitant chemoradiation therapy (CRT) is a frequent treatment modality applied to several types of solid tumors and has improved cancer treatment. The primary clinical rationale supporting these types of treatment is the role of chemotherapeutic drugs as radiosensitizers. Platinum chemotherapeutic drugs (Pt-drugs) including cisplatin, carboplatin and oxaliplatin are frequently administered in CRT for the treatment of upper aerodigestive tract, genitourinary and colon malignancies (1). Although it has been shown that the combination of Pt-drugs and radiation improve treatment outcome, the optimum parameters for CRT with Pt-drugs and the underlying mechanisms of their synergistic action remain the subject of active investigation.

Cisplatin has significant activity against several forms of neoplasm, including ovarian, cervical, head and neck, and non-small-cell lung cancer. However, side effects and tumor resistance to the drug have limited its applications (2). Carboplatin has less systemic toxicity than cisplatin. The types of cancer that can be treated by carboplatin are similar to those of cisplatin and it has often replaced cisplatin. Oxaliplatin has a different pattern of sensitivity, a safer toxicity profile and activity against cisplatin-resistant cancer. It is a standard chemotherapeutic drug for treatment of colorectal cancer. After entry of the Pt-drug molecules into the cell, they subsequently convert to chemically reactive forms due to hydrolysis and react with DNA via ligand exchange at the platinum atom to form Pt-drug-DNA adducts (Pt-adducts) including intra and interstrand cross-links (CL) and monofunctional binding to guanine (3). Formation of these CLs leads to the distortion of DNA conformation by unwinding, bending and destabilization of the double helix (4). In addition to affecting transcription and replication processes, the Pt-adducts are believed to specifically inhibit DNA repair of radiation-induced lesions and enhance radiation damage to DNA (5).

The biological impact of ionizing radiation results from the induction of a variety of lesions, predominantly via energy deposition into the DNA itself (direct effect) and its surrounding molecular environment, consisting mostly of water molecules (indirect effect). The energy deposition generates intermediate species including ions, radicals, excited molecules and free electrons in nanoscale volumes that subsequently interact with cellular DNA. The most numerous of these species are non-thermal secondary electrons. Most of
the latter have energies below 30-eV and a most probable energy of about 10-eV (6). These LEEs, which carry most of the primary radiation energy, have a mean free path of a few angstroms. Thus they can deposit all of their energies inside DNA and induce damage including single- and double-strand breaks (SSB and DSB), base release and modification via resonance scattering mechanisms (7).

Pt-drugs can enhance radiation damage to DNA by increasing the number of the secondary reactive species generated by primary radiation and/or by sensitizing DNA towards these species. In the indirect effect of radiation, cisplatin sensitizes DNA to hydroxyl radicals and hydrated electrons resulting in the enhanced formation of SSB and DSB (8). Theoretical and experimental studies have also reported that both LEEs and prehydrated electrons can interact with cisplatin and release the chlorine atom from its molecule via dissociative electron attachment (DEA) (9,10). Such an indirect effect of radiation produces reactive radicals of cisplatin, which can damage cellular components, including DNA (11,12).

In our laboratory, Zheng et.al. studied direct interaction of LEEs with dry solid films composed of plasmid DNA modified by cisplatin and observed an enhancement in the formation of SSB and DSB (13). Later, Rezaee et.al. showed that the conditions of reaction of Pt-drugs with DNA have substantial effects on the chemical stability of DNA, making it sensitive to the manipulations required for sample preparation (14). Minimizing the adverse effects of platination on DNA integrity, we here investigate the relative efficiency of the Pt-drugs cisplatin, carboplatin and oxaliplatin in DNA sensitization to the direct effects of ionizing radiation, with electrons of 10-keV and 10-eV. These two electron energies can represent the direct effect of high-energy ionizing radiation and the secondary LEEs on DNA in radiotherapy. Comparing the results obtained with 10-keV electrons with those of 10-eV, which constitute a major product of ionization, allows us to determine the role of LEEs in the DNA radiosensitization. The optimal DNA radiosensitivity respecting the quantity of Pt-adducts and possible mechanisms responsible for radiosensitisation are determined by measuring the yield of different types of damages and calculating enhancement factors (EFs) as a means of comparing radiosensitization efficiencies. Assuming that the main target in radiotherapy is nuclear DNA, the present results provide guidance for improving CRT.
MATERIALS AND METHODS

Sample Preparation. Plasmid DNA (pGEM-3Zf-) was extracted from E-Coli and mixed with the solutions of Pt-drugs including cisplatin, carboplatin and oxaliplatin. According to the kinetics of binding Pt-drugs to DNA (Fig. 1e-3e) measured by inductively coupled plasma-mass spectroscopy (ICP-MS), samples of Pt-drug-DNA complexes were prepared at various concentration ratios between 1:1 and 64:1 and deposited onto tantalum substrate (S1 supplement).

Sample Irradiation. The nanoscale films were irradiated with electrons in a home-made laboratory apparatus (Fig. e4). The DNA films were individually irradiated with electrons of either 10- or 10,000-eV for periods between 5 s and 16 min.

Qualification of DNA Damages. After irradiation, the films were immediately retrieved from the apparatus and dissolved in TE buffer from their substrate with 95-98% efficiency. The relative percentage of the different structural forms including supercoiled, nicked circular (SSB), linear (DSB) and interduplex CL in each DNA and Pt-drug-DNA sample was obtained by agarose gel electrophoresis. The amount of each structural form of the DNA was then analyzed by ImageQuant (Molecular Dynamics) software (14).

Calculation of the yields of DNA damages. The yield for electrons induced SSB, DSB and interduplex CL (i.e., dimers of two circular forms of plasmid) were derived from the initial linear slopes of the respective exposure–response curves (S2 Supplement). In addition, ratios of the yields from irradiated the Pt-adduct-DNA molecules to those of unmodified DNA were determined as EFs. These factors represent the radiosensitivity index of Pt-drugs in the induction of SSB, DSB and interduplex CL by ionizing radiation and LEEs.

RESULTS AND DISCUSSION

Relative radiosensitization efficiency of Pt-drugs

Table 1 presents the yields of SSB, DSB and interduplex CL in DNA induced by 10-keV and 10-eV electrons in unmodified DNA and DNA containing on average of two Pt-adducts per plasmid (i.e., one Pt-drug per 1600 bp). Assuming a Poisson distribution for the
binding of Pt-drugs to the plasmids, we expect about 13% of plasmids with no adducts, 74% with 1-3 adducts, and 14% with more than three adducts. Fig. 1 compares the EFs for cisplatin, carboplatin and oxaliplatin. The presence of Pt-adducts substantially enhances the formation of DSB and interduplex CL by electrons, particularly for 10-eV, whereas for SSB formation this enhancement is at the most 50%.

Photons and electrons of 0.2–20MeV are standard beams in radiotherapy. The interaction of such photons with biomolecules via Compton and pair production produces electrons with a wide energy distribution. Energy deposition by the high-energy electrons into biological matter can be calculated using the Born approximation, in which the interaction between electron and matter leads to individual localized-molecular collisions, with negligible momentum transfer, separated by mean free paths much larger than atomic dimensions. Accordingly, electrons with the energies of a few keV to a few MeV interact with matter essentially via the same fundamental process. Thus, 10-keV electrons can generally be considered to represent the direct effects of high-energy ionizing radiation on DNA in radiotherapy. The relative radiosensitization efficiency of Pt-drugs can therefore be discussed in terms of the results of 10-keV irradiation.

Table 1. Yields of DNA damage (x10^-8 damage/Gy/bp) for 10-keV (a) and 10-eV (b) electron irradiation of DNA with and without modification by Pt-drugs.

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>DNA Damage</th>
<th>Pure DNA</th>
<th>Cisplatin-DNA</th>
<th>Carboplatin-DNA</th>
<th>Oxaliplatin-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SSB</td>
<td>145.3 ± 23.6</td>
<td>152.2 ± 26.5</td>
<td>160.4 ± 18.6</td>
<td>151.4 ± 18.9</td>
</tr>
<tr>
<td>10 keV</td>
<td>DSB</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>4.5 ± 0.6</td>
<td>6.8 ± 1.1</td>
<td>7.3 ± 0.3</td>
<td>7.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>SSB</td>
<td>78.7 ± 16.2</td>
<td>110.1 ± 15.8</td>
<td>120.4 ± 21.7</td>
<td>114.3 ± 26.3</td>
</tr>
<tr>
<td>10 eV</td>
<td>DSB</td>
<td>1.2 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CL</td>
<td>3.3 ± 0.9</td>
<td>7.2 ± 2.1</td>
<td>10.2 ± 1.6</td>
<td>13.5 ± 4.6</td>
</tr>
</tbody>
</table>

* SSB: single-strand break; DSB: double-strand break; CL: interduplex cross-link
Fig. 1. Enhancement factors in the yields of SSB, DSB and interduplex CL induced by 10-keV (a) and 10-eV (b) electrons in the presence of cisplatin, carboplatin and oxaliplatin. 

* indicates a P-value < 0.05, when the different Pt-drug-DNA complexes are compared to unmodified DNA.

† denotes a P-value < 0.05, when the different Pt-drug-DNA complexes are compared to each other.
For 10-keV electron-irradiated DNA, there is a 1.7-fold increase in the yield of DSB in the presence of carboplatin and oxaliplatin adducts. This factor is reduced to 1.3 for DNA modified by cisplatin. Additionally, the enhancements in DSB yields for DNA modified by either carboplatin or oxaliplatin are significantly larger than that observed in the presence of cisplatin (P-values: 0.0017 and 0.0365, respectively). Similarly, all Pt-drugs sensitize DNA to the interduplex CL formation by 10-keV electrons. Both carboplatin and oxaliplatin enhance the radiation-induced interduplex CL by a factor of 1.6, compared to 1.4 in the case of cisplatin. In contrast to the DSB yield, there is no substantial difference in the yields of interduplex CL between the three types of modified DNA. Since DSB results from two separate lesions in DNA, these findings suggest that the Pt-drugs preferentially enhance the formation of cluster damage to DNA by ionizing radiation. The enhancement is larger in the presence of carboplatin and oxaliplatin relative to cisplatin. The presence of cluster damages together with Pt-adducts is expected to be extremely toxic for cells owing to the difficulty in performing error-free repair of such locally multiply damaged sites.

Pt-drugs bind to DNA to form mainly intrastrand and interstrand CLs with the purine bases, particularly guanine (4). Despite the similarity in the types of DNA adducts, each Pt-drug produces different proportions of the specific adducts. The major carboplatin adduct, for instance, is reported to be 1,3-d(GpNpG) intrastrand CL, whereas cisplatin and oxaliplatin mostly form 1,2-d(GpG) intrastrand CL (15). The presence of the diaminocyclohexane ligand in oxaliplatin has been shown to lead to several conformational differences between DNA adducts formed by oxaliplatin and cisplatin, predominantly due to the interaction between diaminocyclohexane ligand and DNA constituents (15). Although subtle, such differences distinctively change the conformation of DNA: the carboplatin adduct unwinds DNA to a higher degree than the cisplatin adduct (23° versus 13°, respectively), and oxaliplatin bends DNA less than cisplatin (15). These different alterations in the DNA conformation suggest that each Pt-drug may affect the chemical and physical properties of modified DNA in a distinctive manner, and hence the interaction of LEEs with DNA, as observed in our results. Therefore, it is suggested that the type of Pt-adduct plays a vital role in the DNA sensitization towards ionizing radiation.

In the clinic, carboplatin is an appropriate alternative to cisplatin, owing to its lower side effects and a spectrum of activity similar to cisplatin. Since carboplatin has a less
labile leaving group (cyclobutane di-carboxylate) than chloride and oxalate ligands in cisplatin and oxaliplatin, respectively, it shows a lower reactivity to both hydrolysis and sulphur-containing molecules such as glutathione and metallotheonines, thus permitting the administration of larger doses and greater accumulation of carboplatin inside the cell nucleus relative to cisplatin and oxaliplatin (2). For equal number of Pt-adducts, our results indicate that carboplatin, when administered concurrently with radiation, would be expected to be superior to cisplatin due to its greater efficiency in the induction of cluster damages to DNA, predominantly DSB. Hence, the radiosensitization effects of carboplatin are expected to be greater than those of cisplatin in the clinic. Oxaliplatin has a different spectrum of activity relative to cisplatin and carboplatin, and at the same concentration, it has a lower reactivity than the two other Pt-drugs to DNA, leading to lower levels of adducts, although its cytotoxicity is similar to cisplatin (16). Our results indicate that oxaliplatin further enhances the formation of DSB compared to cisplatin by a factor of 1.3 for 10-keV electron radiation. The superiority of oxaliplatin to cisplatin for radiosensitization, on a ‘per adduct’ basis, suggests that one should obtain a similar radiosensitization of tumour cells with lower levels of oxaliplatin adducts.

The role of LEEs in the radiosensitization of modified DNA

Electrons damage DNA through both resonant and non-resonant processes. The latter are single- or multiple-event processes including ionization and dissociative electronic excitation, whereas the former is a single-event process, which induces DNA damages by the formation of a local transient anion and its decay into the dissociative electronic excitation or dissociative electron attachment (DEA) channels. These electron resonances constitute the dominant interaction of electrons with energies lower than 15 eV (LEEs) with DNA. To determine the contribution of resonant processes towards the observed enhancements of DNA damage by high-energy radiation in the presence of Pt-drugs, solid films composed of either unmodified or modified DNA were also irradiated with 10-eV electrons.

As shown in Table 1 and Fig. 1, 10-eV yields of DSB increase by factors of 3.1, 2.5, and 2.4, respectively, when carboplatin, cisplatin and oxaliplatin are bound to DNA (P-value < 0.001). Moreover, the enhancement of DSB is greater with carboplatin than with
either cisplatin or oxaliplatin (P-value: 0.013, 0.008, respectively). The yields of interduplex CL induced by 10-eV electrons are also enhanced significantly by factors of 2.2, 3.1 and 4.1 in the presence of cisplatin, carboplatin and oxaliplatin, respectively, (P-value < 0.05). Since LEEs constitute a major portion of the secondary species generated by high-energy radiation, these findings suggest that, in the presence of Pt-adducts, LEEs play a major role in the enhancement of DNA damage, particularly cluster damages.

Fig. 2 shows the exposure-response curves for the formation of DSB and interduplex CL in modified DNA irradiated with 10-eV electrons. These curves exhibit a linear behaviour, which suggests that a single-hit process is responsible for the LEEs-induced damages. For interduplex CL, it is reasonable to suggest that a reactive specie formed on one DNA molecule may attack an adjacent molecule, thus one would expect a linear exposure-response curve. However, the interaction of only one LEE with DNA also leads to DSB, which are due to two separate lesions. At 10-eV, the incident electrons essentially break the bonds between DNA constituents via core-excited resonances (7, 17). Dissociative TNI can rupture chemical bonds between DNA constituents via DEA; alternatively they may decay via electron autodetachment resulting in the departure of an electron with lower kinetic energy and the formation of an electronically excited neutral molecule that may itself dissociate into various fragments (18). In the presence of Pt-adducts, we hypothesize that dissociation of one TNI formed at the phosphate group of DNA may lead to the formation of the second lesion via two mechanisms: subsequent formation of a TNI and mechanical stress. The former may occur when the TNI decays via electron autodetachment and leaves the molecular group in an electronically excited dissociative state. In this manner, the phosphate group can dissociate to produce a SSB, and the departing electron can be recaptured by the Pt-adduct on the adjacent strand to form a subsequent TNI, which dissociates into an anion and a radical. The mechanical stress results from the modification of DNA conformation due to the Pt-adducts, which reduces the chemical stability of DNA and weakens certain bonds, particularly at the site of DNA platination. Thus, during the conversion of supercoiled to circular plasmid, which normally leads to a SSB, other weak bonds could rupture resulting in the formation of a second lesion.
Fig. 2. Exposure-response curve for the formation of DSB (a,b) and interduplex CL (c,d) by either 10-eV or 10-keV electrons in DNA modified by cisplatin, carboplatin and oxaliplatin. Data are means ± standard deviation from five measurements. They have been fitted by employing a least-squares regression analysis.

Such mechanical stress can also be responsible for the observed differences in the $EFs$ among the Pt-drugs. Structural alterations of the double helix of DNA induced by carboplatin (due to the formation of 1,3-d(GpNpG) intrastrand CL), for example, are more
severe than those induced by cisplatin and oxaliplatin (i.e., the formation of 1,2-d(GpG) intrastrand CL) (15). This difference results in a greater perturbation of the chemical bonds between DNA components at the platination site. Such perturbation could enhance the formation of TNI and dissociation of the chemical bonds.

**Dependence of DNA radiosensitization on the number of Pt-adducts**

Fig. 3 shows the EFs for the formation of DSB, interduplex CL and SSB by 10-eV electrons as a function of the number of Pt-adducts. The curves of these EFs for DSB and interduplex CL formations exhibit a biphasic behaviour, with an initial steep slopes ($S_1$) of about $(4.0-9.5) \times 10^3$ up to the $0.31 \times 10^3$ Pt-adducts per nucleotide and a final slopes of $(0.5-0.7) \times 10^3$ (Table 2). Hence, *optimum radiosensitization of DNA, in terms of damage per Pt-adduct lies below ratios of $0.31 \times 10^3$ Pt-adducts per nucleotide.*

Intrastrand CLs are the primary Pt-adducts observed in short synthetic DNA and in cultured cells. However studies on the platination of plasmid DNA show that at a low concentration of Pt-drugs (less than $0.5 \times 10^3$ Pt-molecules/nucleotide), interstrand CLs are the most probable adducts in the supercoiled form, while intrastrand CLs are the most probable in the relaxed and linear DNA forms (19). This concentration of Pt-drugs is very close to that of which the EF curve changes slope in the present study (Fig. 3). These findings suggest that *the substantial difference observed in the radiosensitivity of modified DNA at the ratios beyond $0.31 \times 10^3$ Pt-adducts per nucleotide may depend on the type of Pt-adduct (e.g., interstrand versus intrastrand crosslinks).* This ratio however has a value of one or two orders of magnitude higher than those measured from the malignant tissue of the patients typically treated with Pt-drugs in clinical trials. Since Pt-drugs, particularly cisplatin and oxaliplatin have severe side effects such as neurotoxicity, renal and gastrointestinal toxicity, it is impossible to increase the dose of the drugs in clinical applications. In contrast, the ratio of Pt-drugs to DNA in the cancer tissue of the patients treated with liposomal Pt-drugs, i.e., encapsulation of the drug into nanoparticle formulation, is similar to our proposed ratio resulting in the optimal radiosensitization (20).
Fig. 3. Enhancement factors in the yields of SSB, DSB and interduplex CL as a function of the number of Pt-adducts per nucleotide for DNA modified by cisplatin (a), carboplatin (b) and oxaliplatin (c) irradiated with 10-eV electrons. The fitted lines are based on a least-square regression analysis.
Table 2. Enhancement Factors for the induction of SSB, DSB and interduplex CL as a function of the number of Pt-adducts per nucleotide. $S_1$ and $S_2$ denote the slopes of the fitted lines to the Enhancement Factor curves for the DNA damages presented in Fig. 3 at ratios less and more that $3.1 \times 10^4$ Pt-adducts per nucleotide, respectively. $R_{1,2}$ is the ratio of $S_1$ to $S_2$.

<table>
<thead>
<tr>
<th>DNA Damage</th>
<th>Pt-drug</th>
<th>$S_1$ ($\times 10^3$)</th>
<th>$S_2$ ($\times 10^3$)</th>
<th>$R_{1,2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSB</td>
<td>Cisplatin</td>
<td>0.51 ± 0.03</td>
<td>0.51 ± 0.03</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Carboplatin</td>
<td>0.57 ± 0.04</td>
<td>0.57 ± 0.04</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Oxaliplatin</td>
<td>0.56 ± 0.06</td>
<td>0.56 ± 0.06</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>DSB</td>
<td>Cisplatin</td>
<td>4.8 ± 0.2</td>
<td>0.54 ± 0.07</td>
<td>8.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Carboplatin</td>
<td>6.7 ± 0.8</td>
<td>0.65 ± 0.1</td>
<td>10.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Oxaliplatin</td>
<td>4.5 ± 0.6</td>
<td>0.59 ± 0.1</td>
<td>7.6 ± 1.6</td>
</tr>
<tr>
<td>CL</td>
<td>Cisplatin</td>
<td>3.84 ± 0.2</td>
<td>0.65 ± 0.06</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Carboplatin</td>
<td>6.4 ± 0.9</td>
<td>0.62 ± 0.08</td>
<td>10.3 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Oxaliplatin</td>
<td>9.5 ± 2.6</td>
<td>0.67 ± 0.03</td>
<td>14.2 ± 3.9</td>
</tr>
</tbody>
</table>

* SSB: single-strand break; DSB: double-strand break; CL: interduplex cross-link

CONCLUSION:

The presence of Pt-adducts preferentially enhances the formation of DNA cluster damages including DSB and interduplex CL by ionizing radiation and LEEs produced by the direct effect of ionizing radiation. Despite similarities between Pt-drugs in the enhancement of the DNA lesions, carboplatin and oxaliplatin have a higher efficiency than cisplatin. Since the reactive forms of the Pt-drugs are identical between cisplatin and carboplatin, and similar to oxaliplatin, the type of Pt-adducts must be responsible for the observed different efficiencies in DNA radiosensitization by Pt-drugs. The yields and EFs for the formation of cluster lesions by 10-eV electrons are larger than those by 10-keV electrons, suggesting that LEEs are the main secondary species responsible for the enhancement of DNA damage in the presence of Pt-drugs. In addition, the induction of cluster lesions by 10-eV electrons results from a single-hit process as deduced from the linearity of the exposure-response curves. Moreover, radiosensitization enhancement versus
quantity of Pt-adducts is bi-phasic with a change in slope at a ratio of $3.1 \times 10^{-4}$ Pt-adducts per nucleotide. It appears that the radiosensitization by Pt-drugs depends considerably on the type of Pt-adducts.

For clinical applications, therefore, it is reasonable to infer that the effectiveness of radiation beams generating more LEEs would be higher in Pt-based CRT. Owing to the greater ionization density, the radiation beams with high linear energy transfer (LET), such as alpha, proton and heavy ion beams, produce a large number of LEEs in their tracks compared to low LET radiation (e.g., photons and electrons), hence they should be more efficient in Pt-based CRT.
REFERENCES:
S1 SUPPLEMENT

Sample Preparation and Manipulation

Plasmid DNA (pGEM-3Zf-, 3199 base pairs) was extracted from *E.coli* JM109, purified with a HiSpeed plasmid Maxi kit (QIAGEN) and redissolved in TE buffer (10 mM Tris, 1 mM EDTA) with pH 8. Prior to use, DNA was cleaned up from TE by applying a home-made microcolumn of Sephadex G-50 resin on a bed of glass beads. The concentration of DNA and its purity were then determined by measuring the ratio of ultraviolet (UV) absorption of DNA and the contaminating protein at 260 and 280 nm, respectively, with a Synergy HT-I spectrophotometer. For the plasmid DNA used in this study, this ratio was 1.99 which corresponds to purity greater than 95% (1). Analysis of these plasmids by agarose gel electrophoresis showed that the purified plasmid DNA consisted of 96% supercoiled, 2% concatemeric, and 2% nicked circular forms.

Cisplatin, carboplatin and oxaliplatin were purchased from Sigma-Aldrich with stated purities of higher than 98% and used without further purification. Their solutions were prepared in ddH2O based on their molar solubility. Tris buffer was also added to the solution of DNA in ddH2O at a ratio of one tris compound per nucleotide and the final solution was split into several parts. According to the kinetics of binding cisplatin, carboplatin and oxaliplatin to DNA (Fig. 1e-3e), the solution of each Pt-drug was added to one part of the latter DNA solution at different initial concentration ratios between Pt-drug molecules and plasmid DNA. To maintain the integrity of plasmid DNA, the reaction of DNA platination was performed at the room temperature for a few hours in the dark (2). Under this condition, the difference in the percentage of supercoiled configuration (i.e., a measure for intact DNA) for Pt-DNA and pure DNA samples was less than 2%, indicating that the formation of Pt-DNA adducts lead to the negligible formation of strand breaks in the plasmid DNA. After appropriate incubation time with respect to the kinetics, the unbound Pt-drug molecules, Tris compounds and any complexes of Tris with Pt-drugs were separated from the Pt-drug-DNA solution by gel filtration with a Sephadex G-50 medium. Such filtration was expected to produce clean solutions of Pt-DNA in ddH2O. The final solutions were Pt-drug bound to DNA with a known ratios of Pt-drugs bound to DNA (Pt-drug-DNA), including 1:1, 2:1, 4:1, 8:1, 16:1, 32:1 and 64:1 as determined by inductively
coupled plasma mass spectroscopy (ICP-MS). At each Pt-drug-DNA ratio, a pure DNA sample was also prepared and kept under the same conditions as the Pt-drug-DNA sample.

**Nanometer-scale solid films**

The frozen-dried samples (films) deposited on tantalum (Ta) had a ring shape of average radius $r$. Assuming a density ($\rho$) 1.71 g.cm$^{-3}$ for the plasmid extracted from *E. coli*, the average thicknesses ($h$) of different groups of DNA films were determined by applying the formula:

$$m_{DNA} = \rho V = \rho Ah = \rho (\pi r^2)h$$  \hspace{1cm} (I-1)

Here, $m_{DNA}$ is the mass of DNA in each film, and $A$ the area of the film. Thus, by measuring $A$, it was possible to deduce $h$, the average thickness of the film, within a 30% error; the latter arose principally from the uncertainty on $A$. With respect to the range of the electrons, 10 and 400 nm DNA film thicknesses were prepared for irradiation by 10-eV and 10-keV electrons, respectively, assuming minimum aggregation of plasmids within the solid films. Accordingly, films contained 210 and 4200 ng of DNA within an area of 0.1256 cm$^3$. It is important to both monitor the incident beam current during electron impact and shield these DNA films placed in the UHV system from scattered electrons as much as possible to avoid charging the irradiated film. The film thicknesses were chosen according to the efficient range of 10-eV and 10-keV electrons, so as to insure minimal generation of secondary LEEs from the Ta substrate. In this manner, substantial DNA damage from these short-range LEEs could be avoided.

Such a dried DNA still contains, on average, 2.5 water molecules per nucleotide, which remain in the double helix, as an integral part of the DNA structure (3). Any damage to DNA by such water molecules is considered as the direct effect of ionizing radiation (4, 5).

**References**


Calculation of Yields of Damage

For each incident electron energy, the exposure-response curves for the different plasmid configurations including circular, linear and interduplex CL were obtained for the unmodified and all modified DNA samples. In these curves, the relative percentages of the different DNA forms were plotted as a function of electron exposure ($\varepsilon$). The latter is the number of incident electrons impinging on the DNA sample and is given by:

$$\varepsilon = J t A = \varphi_0 A,$$

(II-1)

Where $J$, $t$, $A$ and $\varphi_0$ are the electron current density, irradiation time, the area of the DNA film (sample) and the fluence of incident electrons, respectively. Since the parameters $J$ and $A$ had fixed values in the present study, the exposure $\varepsilon$ was only adjusted by the irradiation time $t$. At 10 eV, for example, the electron beam current was 2 nA, giving a current density of $9.95 \times 10^{10}$ electrons.cm$^{-2}$.s$^{-1}$. By knowing $A$, it is possible to calculate the number of electrons impinging into the sample per second.

Each exposure-response curve was obtained by irradiating 6-8 identical samples at increasing radiation exposure. A sample kept under the same conditions as the irradiated samples but never irradiated with electrons served as a control sample. This procedure was repeated five times for each incident electron energy. With respect to four different samples (pure DNA, cisplatin-DNA, carboplatin-DNA and oxaliplatin-DNA), about 140 samples were irradiated for each incident electron energy at different exposures, for a total of about 700 samples irradiated for the measurement of the DNA damages in both unmodified DNA and the modified DNA at the different ratios of Pt-drugs to plasmid. The yields ($Y'$) of the formation of circular, linear and interduplex CL were then calculated from linear least-square fits to the initial slopes of the exposure-response curves for each incident radiation. $Y'$ represents the number of the DNA damages per each incident electron. Then $Y'$ was converted to yield of damage per absorbed dose (Gy) for each base pair, denoted by $Y$, to compare the efficiency of electrons of different energies in the enhancement of the damage formation in the modified DNA.

For electrons of 10 keV, the yield $Y_{10keV}$ (number of damage.Gy$^{-1}$.bp$^{-1}$) is given by:
\[ Y_{10\text{keV}} = \frac{Y'_{10\text{keV}}}{N_{bp}} \left[ \frac{m}{C(dT/\rho dx)_c \rho h} \right], \quad \text{(II-2)} \]

where \( C \) is an energy conversion coefficient of \( 1.602 \times 10^{-19} \text{ eV J}^{-1} \), \( Y' \) is the yield of damage per incident electron in each plasmid DNA, and \( N_{bp} \) is 3197, i.e., the number of base pair in the plasmid pGEM3Zf(-). The bracket factor converts the incident electron energy to the absorbed dose in a 400 nm (\( h \)) thick solid film composed of 4.2 \( \mu \text{g} \) plasmid DNA (\( m \)) with density \( \rho \) of 1.71 g cm\(^{-3} \) and the collision stopping power \( (dT/\rho dx)_c \) of 21.4 MeV cm\(^2 \) g\(^{-1} \) for 10-keV electrons in the solid DNA (1).

The yield of damage for 10-eV electrons is calculated from:

\[ Y_{10\text{eV}} = \frac{Y'_{10\text{eV}}}{f_i N_{bp}} \left[ \frac{m}{CE_i \left(1 - e^{-h/\lambda}\right)} \right], \quad \text{(II-3)} \]

where the \( f_i \) is a correction factor for the effect of film charging on the initial slope of the exposure-response curve and \( \lambda \) is the attenuation length of 10-eV electrons inducing DNA strand breaks passing through a solid film of plasmid DNA. For electrons of incident energy \( E_i \) of 10 eV impinging on a 10 nm (\( h \)) thick film of 210 ng (\( m \)) plasmid DNA, \( f_i \) and \( \lambda \) are 0.64 and 10.4 nm, respectively (2).

References
Fig. e1. Kinetics of binding cisplatin to plasmid DNA at three different initial ratios of cisplatin to DNA in the solution: (a) 200:1, (b) 80:1, and (c) 20:1. The curves show the quantity of bound cisplatin per DNA molecule at different incubation times at room temperature. Data in a – c are means ± SD (standard deviation) from three measurements. The dash lines are exponential fits to the data.
Fig. e2. Kinetics of binding oxaliplatin to plasmid DNA at three different initial ratios of oxaliplatin to DNA in the solution: (a) 200:1, (b) 80:1, and (c) 20:1. The curves show the quantity of bound oxaliplatin per DNA molecule at different incubation times at room temperature. Data in a–c are means ± SD (standard deviation) from three measurements. The dash lines are exponential fits to the data.
Fig. e3. Kinetics of binding carboplatin to plasmid DNA at three different initial ratios of carboplatin to DNA in the solution: (a) 200:1, (b) 100:1, and (c) 30:1. The curves show the quantity of bound carboplatin per DNA molecule at different incubation times at room temperature. Data in a – c are means ± SD (standard deviation) from three measurements. The dash lines are exponential fits to the data.
Figure e4. Schematic view of UHV electron irradiator chamber and the principal components. The assembly consists essentially of a rotatable circular platform (F) connected to a rotary drive (E) and two types of electron guns, i.e., a low-energy electron gun (C) mounted on a linear drive (G) and a fixed high energy electron gun (H) (Kimball Physics Inc.). The former produces a beam adjustable in energy between 0.5 and 1000 eV, with the spot size of the beam varying between 2 and 50 mm at working distances of 10 and 50 mm. The latter generates an electron beam adjustable within the 0.5 - 20 keV energy range and in spot sizes at the fixed working distance of 20 cm. The spot size is controlled by an electrostatic lens system using a triode configured electron source with a control grid aperture, i.e., a symmetric einzel focus lens, and four plates centering deflection system. In the present experiment, the spot size of both electron beams was set to irradiate an area of about 0.9 cm² which was 7 times larger than the DNA film. A 0.3 mm wide slit followed by a Faraday cup detector and a phosphorescent screen were used to calibrate...
the electron current and its spatial distribution. For irradiation by low- and high-energy electrons, the DNA films were directly transferred to the UHV chamber, which can be opened by a quick access port (D) from the inside of a glove box (B), kept under a dry N₂ atmosphere during lyophilization of a DNA solution on the substrate. The UHV chamber was then evacuated for 24 hours by a hydrocarbon-free turbomolecular pump to a pressure of 5 x 10⁻⁹ Torr measured by an ion gauge (A) at room temperature.
II.4. Fourth Article: A single subexcitation-energy electron can induce a double strand break in DNA modified by platinum chemotherapeutic drugs

Authors: Mohammad Rezaee, Elahe Alizade, Darel J. Hunting, Léon Sanche

Status: Submitted Paper to Angewandte Chemie International Edition

Contributions: Mohammad Rezaee designed and performed research, analyzed data for low-energy electron experiments, and wrote manuscript. Dr. Elahe Alizadeh performed experiments and analysed data for X-rays irradiation. Professor Leon Sanche and Professor Darel Hunting supervised the project, advised on the design and data interpretation, and edited the manuscript.
A single subexcitation-energy electron can induce a double strand break in DNA modified by platinum chemotherapeutic drugs

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ABSTRACT
Radiosensitization of malignant cells is the clinical rationale for the use of platinum-based concurrent chemoradiotherapy for cancer treatment; however the specific mechanisms of radiosensitization still remain to be determined. The present study demonstrates that the abundant of near zero-eV (0.5 eV) electrons, created by ionizing radiation, induce double strand breaks (DSB) in supercoiled plasmid DNA modified by platinum anticancer drugs (Pt-drugs), but not in unmodified DNA. They do so more efficiently than with other types of radiations including soft X-rays and 10-eV electrons. Furthermore, these near-zero-eV electrons are more efficient than soft X-rays and 10-eV electrons in forming single strand breaks (SSB) in DNA, particularly when the latter is modified by Pt-drugs. The formation of DSB by 0.5-eV electrons is found to be a single-hit process owing to the linearity of the respective exposure-response curves. Since the energy of these electrons is well below that required for ionization and electronic excitation of organic molecules, dissociative electron attachment (DEA) via shape resonances must be responsible for the observed damage in modified DNA. It appears that the presence of Pt-DNA adducts modifies the electronic properties of DNA components at the site of platination, which could enhance both the formation of a temporary anion and its subsequent decay into the DEA channel. These findings provide revealing insights into the radiosensitization mechanism of Pt-drugs that may have implications in the development of more efficient platinum-based concurrent chemoradiation therapy. In addition, our finding suggest that in situ sources of subexcitation-energy electrons, such as Auger-electron emitting radionuclides could efficiently enhance DSB formation in DNA modified by Pt-drugs in malignant cells.

INTRODUCTION
Concurrent administration of platinum-based chemotherapeutic drugs (Pt-drugs), including cisplatin, carboplatin and oxaliplatin, with radiation therapy significantly improves patient survival for a variety of solid tumors such as cervical, lung, head and neck cancers (1-4). The main purpose of concomitant treatment lies in the role played by the Pt-drugs as radiosensitizers that result in the improvement of local therapy, while also providing systemic therapy (5, 6). For such modality combination that is now a standard treatment method in clinic, several biological consequences, including inhibition of repair
mechanisms and preferential radiation-induced DNA lesions in the vicinity of Pt-DNA adducts, have been suggested; however the optimum schedule for the combination resulting in the synergistic toxicity in tumor cells has not yet been determined. Thus, to identify the synergistic combination, elucidation of the entire sequence of physical and physicochemical events leading to the biological effects, and understanding of their nanoscopic mechanisms are essential.

Pt-drug molecules enter a cell via both passive diffusion and active transporters; then they convert to chemically reactive forms as a result of hydrolysis reactions (7). Subsequently, they react with cellular components, including mainly DNA, RNA and proteins, via ligand exchange at the platinum atom (8, 9). Binding of Pt-drugs to DNA causes a local distortion of the DNA conformation and affects physical and chemical properties of the molecule, particularly at the site of platination (10-12). These modifications are believed to enhance the effect of radiations on the modified DNA by perturbing processes, which occur during the physical and physicochemical stages of the interaction of radiation with DNA (13, 14). At these very early stages measured in femtoseconds, the platinum atom is proposed to play a vital role in the enhancement of radiation-induced damage to DNA (15).

Given the high atomic number of platinum, inner-shell ionization of this atom by primary or secondary radiation results in the generation of numerous low energy Auger electrons which subsequently interact with DNA and induce cluster lesions (15). However, this mechanism is less probable in the routine radiotherapy with high energy photons and electrons since such radiations have energies much higher than the optimal energy for inner-shell ionization of platinum atom and they interact with matter mostly via Compton effects or soft collisions that predominantly cause outer-shell ionization (16). Another important characteristic of the binding of Pt-drugs to DNA associated with radiosensitization is the higher cross section for DNA damage induced by low energy electrons (LEEs) (17). These non-thermal electrons are the most numerous of the secondary species produced along with the primary radiation track and they carry most of the primary radiation energy. The vast majority of these electrons have energies below 30 eV with a most probable energy at about 10 eV (18). Even when formed with energies less than that
required for ionization of DNA, these LEEs can induce damage to DNA via a resonance mechanism (19, 20).

The direct interaction of LEEs with plasmid DNA modified by cisplatin has been shown to increase DNA strand breaks, by 10- and 100-eV electrons compared to unmodified DNA (21). Carboplatin and oxaliplatin have been observed to have higher efficiencies than cisplatin for the enhancement of DNA damage induced by 10-eV electrons, and it has been suggested that LEE-interactions are implicated in the enhancement of damage to DNA by Pt-drugs (Rezaee et. al.). In addition, experimental evidence suggests that LEEs specifically induce cluster damages, such as double strand breaks (DSB) and interduplex crosslink, in supercoiled DNA modified by Pt-drug-DNA adducts (Pt-adducts) (22). At 10 eV, an incident electron is captured by a DNA constituent to form a local transient negative ion (TNI) via core-excited resonance process, which may subsequently rupture chemical bonds via either dissociative electron attachment (DEA) or dissociative electron excitation after electron autodetachment (23).

To determine the contribution and efficiency of these decay channels in the induction of damage to DNA modified by Pt-drugs, we here investigate the effect of electrons with energies below those required for the ionization and electronic excitation of biomolecules (i.e., subexcitation-energy electrons) on supercoiled plasmid DNA in the presence of Pt-drugs including cisplatin, carboplatin and oxaliplatin. The relative efficiency of DNA sensitization by Pt-drugs is determined by measuring the yields of SSB and DSB for bombardment of the same target with either LEEs of 0.5 ± 0.2 eV or 1486-eV X-ray photons. Comparison of these results together and with those of 10 eV (from a previous study) allows us to determine the role of subexcitation-energy electrons and DEA in the radiosensitization of DNA, which is an important step to unravel LEE mechanisms underlying the role of Pt-drugs in DNA radiosensitization.

RESULTS AND DISCUSSION

Near-zero-eV electrons induce strand breaks in DNA modified by Pt-drugs

Fig. 1 presents exposure response curves for the formation of nicked circular and linear DNA corresponding to the formation of SSB and DSB, respectively, in DNA and Pt-drug-DNA films (SI Appendix, Table S2) irradiated with electrons of 0.5 eV. This energy is the
lowest that can be reproducibly attained by our monoenergetic-electron gun under ultrahigh vacuum (UHV) conditions. The latter is essential to prevent contamination of the DNA film surface from environmental impurities which could react with DNA under LEE irradiation.

The platinated plasmid contained an average of two Pt-drugs per plasmid (i.e., one Pt-drug per 1600 base pairs). With this ratio and assuming a Poisson distribution for the Pt-drugs among the plasmids, about 74% of the plasmids should have 1-3 Pt-adducts, 13% should have no adducts and 14% should have more than three adducts. No DSB were observed for the irradiated unmodified DNA, as expected from the reported threshold for DSB induction

**Fig. 1.** Exposure-response curve for the formation of nicked circular (left) and linear (right) DNA configurations corresponding to the SSB and DSB, respectively, by 0.5-eV electrons in DNA unmodified or modified by cisplatin, carboplatin and oxaliplatin. The sample films were deposited on tantalum. The minimum detection limit of a band in the agarose gel electrophoresis is (0.15 ± 0.05)%. Data are means ± SD (standard deviation) from five measurements. They have been fitted by a least squares regression analysis. Dotted lines are guides for the eyes.
of 6-7 eV (24). In striking contrast to unmodified DNA, 0.5-eV electrons induce DSB in the DNA modified by Pt-adducts. Moreover, the initial slope of the exposure-response curve for the formation of SSB is greater in the case of modified DNA than that of the unmodified molecule.

Since the range of 0.5-eV electrons in the condensed DNA has not been determined yet, calculation of the precise deposited energy in the irradiated films and subsequently the yields of damage per absorbed dose for the modified DNA irradiated with these subexcitation-energy electrons are not feasible. However, the minimum values for the yield of damage can be estimated by assuming that the energy of all incident 0.5-eV electrons is entirely absorbed in the 10-nm thick films. With respect to the measured attenuation length and mean free path of LEEs in the solid films of DNA or amorphous ice, this assumption is quite reasonable (25-27). The yields of SSB and DSB in unmodified and modified DNA induced by 0.5-eV electrons are presented in Table 1 along with those induced by 10-eV electrons (22) and 1486-eV X-ray photons (refer to Materials and Method).

Comparison of these minimum yields with those reported for 10-eV electron irradiation shows that the yield for SSB formations in modified DNA is greater for 0.5-eV electrons than those of 10 eV. In addition, the yields of SSB formation in unmodified DNA are significantly larger for electrons of 0.5 eV than for those of 10 eV ($P$ value < 0.05). For the 0.5-eV electron-irradiated DNA, moreover, there are enhancements in the yield of SSB by factors of 1.5, 2.2 and 1.6 in the presence of cisplatin, carboplatin and oxaliplatin, respectively. For DNA modified by carboplatin, this enhancement factor is higher than that of 10-eV electrons (2.2 versus 1.5), whereas the factors are relatively similar for both 0.5- and 10-eV electrons in the presence of cisplatin and oxaliplatin. Given that 0.5-eV electrons are not able to induce DSB in unmodified DNA, it is not possible to determine the enhancement factor for the formation of DSB. These results show that electrons of 0.5 eV are more efficient than those of 10 eV to induce SSB in the DNA modified by Pt-adducts. Furthermore, the efficacy of 0.5- and 10-eV electrons in the formation of DSB is similar in the presence of Pt-adducts. Since 0.5 eV is well below that energy required for the electronic excitation of organic molecules, these findings indicate that DEA via shape resonances is the mechanism responsible for the formation of SSB and DSB in the presence of Pt-adducts.
Table 1. Yields of DNA damage (x 10⁻⁸ damage/Gy/bp) for soft X-rays, 10- and 0.5-eV electron irradiations of DNA with and without modification by platinum anticancer drugs.

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>LET (eV/nm)</th>
<th>Pure DNA</th>
<th>Cisplatin + DNA</th>
<th>Carboplatin + DNA</th>
<th>Oxaliplatin + DNA</th>
<th>Pure DNA</th>
<th>Cisplatin + DNA</th>
<th>Carboplatin + DNA</th>
<th>Oxaliplatin + DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5-eV Electrons</td>
<td>0.05</td>
<td>0</td>
<td>4.4 ± 1.5</td>
<td>6.0 ± 2.3</td>
<td>7.4 ± 3.1</td>
<td>324.9 ± 73.8</td>
<td>487.4 ± 74.5</td>
<td>701.5 ± 44.3</td>
<td>531.7 ± 59.1</td>
</tr>
<tr>
<td>10-eV Electrons (22)</td>
<td>0.32</td>
<td>1.2 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>78.7 ± 16.2</td>
<td>110.1 ± 15.8</td>
<td>120.4 ± 21.7</td>
<td>114.3 ± 26.3</td>
</tr>
<tr>
<td>1.5-keV X-rays</td>
<td>19.2</td>
<td>ND*</td>
<td>4.9 ± 0.8</td>
<td>7.0 ± 1.0</td>
<td>5.4 ± 0.7</td>
<td>199.0 ± 25.3</td>
<td>204.0 ± 26.8</td>
<td>237.2 ± 29.7</td>
<td>215.3 ± 37.3</td>
</tr>
</tbody>
</table>

Subexcitation-energy electrons play a vital role in platinum-based chemoradiation therapy

In radiotherapy, the primary radiation beam, predominantly photons and electrons of 0.2–20 MeV, interacts with biomolecules via several processes, including photo-electric effect, Compton scattering and pair production. These interactions produce electrons with a wide energy distribution, with a maximum at high energy. Energetic electrons transfer their energy to biological matter in localized quanta, separated by mean free paths much larger than atomic dimensions. Since the energy and momentum transfer of the electrons is too small in each interaction (e.g., 24 eV loss on average in condensed DNA) (28), the energy deposition can be described by the first Born approximation (29). Therefore, ionizing particles with energies much larger than the energy lost (i.e., with energies of keV to a few MeV) interact with matter essentially via the same fundamental process. X-ray photons of 1.5-keV interact with DNA via the photoelectric process and generates photoelectrons with energies around 1 keV. Therefore, they can generally be considered to represent the direct effects of radiation on DNA resulting from high energy ionizing radiation in radiotherapy. The relative radiosensitization efficiency of Pt-drugs can therefore be discussed in terms of the results of 1.5-keV irradiation. Furthermore, 1.5 keV photons have high linear energy transfer (LET) as shown in Table 1. Such high LET photons have the higher rate of energy...
transfer per unit path length (i.e., a shorter mean free path) compared to low LET radiation (e.g., high energy photons or electrons), thereby inducing several molecular ionization in a few nanometers of its path length into a scattering medium and subsequently generating numerous secondary LEEs. This characteristic allows performing experiments to compare damage induced by X-rays and LEEs with samples of the same thickness.

Fig. 2 presents exposure-response curves for the formation of nicked circular and linear DNA in unmodified and modified DNA samples deposited on glass substrates (SI Appendix, Table S2). The samples were irradiated with Al Kα 1486-eV X-rays under a dry nitrogen atmosphere. The thickness of the DNA films was 10 nm. It has been shown that the yield of secondary photoelectrons produced in glass and backscattered towards the film due to the interaction of X-ray with the substrate is negligible (30). In addition, gaseous nitrogen does not promote radiation-induced damage to DNA, particularly strand breaks (31). The platinated plasmid contained an average of two Pt-drugs per plasmid. The results clearly show that Pt-drugs substantially enhance the formation of DSB in the X-rays irradiated DNA, but have minimal effect on SSB formation.

For unmodified DNA irradiated with X-rays, the amount of linear plasmid, resulting from a single DSB, was smaller than the detection limit of agarose gel electrophoresis (20 pg/µl). The absorbed X-ray energy within the DNA films in about 0.2% making it technically unfeasible to give doses sufficiently large to induce DSB in unmodified DNA. In contrast, substantial amounts of DSB were observed in all DNA samples modified by Pt-adducts, which were irradiated by X-rays. As presented in Table 1, the yield of DSB formation by X-rays is higher in the presence of carboplatin compared to oxaliplatin and cisplatin. The presence of Pt-adducts has a small effect on SSB formation by X-rays, causing slight increases by factors up to 1.2. These results are in agreement with those obtained with 10-keV electrons (22).

Comparison of the damage yields (Table 1) shows that 0.5-eV electrons are as efficient as X-rays in the induction of DSB in modified DNA. In addition, the efficacy of these near-zero-eV electrons in the formation of SSB is greater than that of X-ray photons. These results are surprising in view of the fact that the LET of 1.5-keV X-rays is much larger, by a factor of about 400, than that of 0.5-eV electrons (Table 1). Since below a few eV no
Fig. 2. Exposure-response curve for the formation of nicked circular (a) and linear (b) DNA configurations corresponding to the SSB and DSB, respectively, by 1486-eV X-ray photons in unmodified DNA or DNA modified by cisplatin, carboplatin and oxaliplatin deposited on glass substrates at room temperature and exposed to 1 bar nitrogen gas. Data are means ± SD (standard deviation) from five measurements. They have been fitted by a least squares regression analysis.
other electron mechanism than DEA can break a strand (32), this finding indicates that *electron resonance are highly efficient in the radiosensitization of DNA by Pt-drugs*. Moreover, since LEEs constitute a major portion of the secondary species produced by high-energy radiation, these results suggest that *electrons of subexcitation energies play an important role in the formation of DNA damage, particularly DSB in the presence of Pt-adducts*.

**DSB formation by 0.5-eV electrons and X-rays in modified DNA is a single-hit process**

DNA DSBs are considered to be the most critical lesion for the biological effects of radiation since, if misrepaired or not repaired, they will have severe consequences for cells such as loss of genetical materials, promotion of genomic instability and cell death (33). A DSB results from damage to the deoxyribose-phosphate backbone of a double helix DNA in two nearby locations leading to the rupture of phosphodiester bonds in two opposite strands (34). In other words, a DSB is essentially formed by at least two separate, but not necessarily uncorrelated, lesions on DNA.

The exposure-response curves shown in Fig. 1 and 2 for the formation of DSB in the modified DNA by electrons of 0.5 eV and X-rays exhibit a linear behaviour. This linearity implies that a single-hit process is responsible for DSB induced by these radiations. Linear dose-response curves for the formation of DSB by the direct effect of photons have also been reported (35, 36). It is possible that an X-ray photon of 1.5 KeV ionizes a DNA constituent via the photoelectric effect, leading to the SSB formation, and the departing photoelectron interacts with another DNA constituent in the opposite strand to induce the second strand break. Our data also indicate that the interaction of only one 0.5-eV electron with DNA leads to the formation of DSB, which requires two separate opposite lesions. In addition to the linearity of the exposure-response curves, comparison of the curves for SSB and DSB formation by 0.5-eV electrons shows that SSB formation, after an initial increase at low doses, reaches saturation, whereas DSBs increase linearly with radiation dose. This difference in the curves suggests that the formation of DSB is independent from SSB formation. To estimate the contribution of single- and double-hit processes to the DSB induction in modified DNA, we employed an analytical method proposed by McMahon and Currell, with modifications (37) (*SI Appendix, Section S.1*). At 2 Gy, i.e., a typical clinical
radiation dose, the total number of DSB resulting from single-hit is much greater than that from double-hit, by factors of 748, 531 and 351 in the presence of oxaliplatin, cisplatin and carboplatin, respectively (SI Appendix, Section S.1 and Table S1). Since DEA is the process responsible for the breakage of chemical bonds in DNA by subexcitation-energy electrons (34), a 0.5-eV electron can break a phosphodiester bond (i.e., formation of an SSB) resulting in an anion and a radical. The latter may attack the opposite strand and induce another SSB, particularly in the presence of Pt-adducts that increase the accessibility of the backbone to the radical by unwinding and bending the double helix (38). Since DSB are not observed at this energy in the absence of Pt-adducts, it seems reasonable to conclude that DEA occurs in the vicinity of these adducts and initiates a series of highly efficient events resulting in the formation of DSB.

The role of DEA in the DNA radiosensitization by Pt-drugs

LEE damage to DNA associated with DEA is widely observed in different types of DNA, including the supercoiled plasmid, synthetic oligonucleotide, and DNA components such as nucleotides, nucleosides and bases (20). In doubly-stranded DNA, the breakage of the backbone occurs via formation of TNI at the phosphate group (i.e., doubly charged PO4) and subsequent dissociation of C-O bond resulting in the rupture of phosphodiester bond (39). In the condensed phase, it is well known that the surrounding molecules largely affect DEA by modifying the energy and life time of TNI, the energy of the dissociation limit and the dynamics of the entire process. For example, the presence of organic ions (e.g., Tris and EDTA) has been shown to protect DNA from the formation of strand breaks by LEEs through modifying the electron capture properties and life time of TNI formed on the phosphate group or base (40, 41). It is also observed that perturbation of the electric field close to a molecule, such as the presence of metals, substantially changes DEA (42, 43).

Dissociative attachment of subexcitation electrons to a molecule or to a basic unit of a large biomolecule (e.g., PO4) proceeds in two steps: (1) the unbound electron is first captured by the molecule or unit through a transition, in the Frank-Condon region, from the neutral electronic ground state into a dissociative anion state (e.g., PO4−), and (2) provided that the lifetime of the anion state is longer than about half a vibrational period of the anion,
the state dissociates into a stable anion and a radical. Therefore, the total cross section for DEA depends on the probability of both electron capture ($\sigma_c$) and anion survival ($P_s$) against electron autodetachment (44, 45), and is given by:

$$\sigma_{DEA} = \sigma_c P_s = \left[ \frac{4\pi^2}{K^2} \frac{\bar{g}}{\Gamma_d} |\chi_v|^2 \right] \exp \left( -\int_{R_e}^{R_c} \frac{\Gamma_a(R)dR}{\hbar v(R)} \right),$$

(1)

where the bracketed factor and the exponential term correspond to $\sigma_c$ and $P_s$, respectively. $K$ is the wave vector of the incident electron in condensed matter (e.g., solid DNA films in our study), $\bar{g}$ is a statistical factor for total spin quantum number and rotational/electronic angular momentum of the initial and final molecular state, $\Gamma_a$ is the autodetachment width of the anion, $\Gamma_d$ is the width of Frank-Condon region, $\chi_v$ is the normalized vibrational nuclear wave function, $v(R)$ is the velocity of the dissociating anionic fragment along its nuclear coordinates; $R_e$ corresponds to the equilibrium internuclear separation of the neutral ground state of the molecule or the electron attaching group, whereas $R_c$ corresponds to the nuclear separation of the anion along the dissociation coordinate, beyond which no autodetachment occurs. We note that the magnitude of the wave factor $K$ in Eq. (1) depends inversely on the wavelength of the incident electron. Hence, $\sigma_c$ for electrons of 0.5 eV is much larger than those of higher energies (e.g., of 10 and 100 eV.)

In the presence of Pt-drugs, unbound electrons are attached in the vicinity of the platination site owing to the high electron affinity of the platinum. Furthermore, this affinity should lower the energy of TNIs formed at the site of platination. Lowering the energy of a TNI reduces the number of decay channels, and hence the parameter $\Gamma_a$ in Eq. (1), which translates into an increase in the magnitude of DEA. The lower energy also increases the survival probability $P_s$ of the anion by reducing $R_c$. Here again, the reduced anion energy increases the DEA cross section. Therefore, even if electrons of 0.5 eV may hardly be able to separate nuclei of a TNI resulting in the dissociation of chemical bonds, particularly the phosphodiester bonds in the DNA backbone, the presence of Pt-adducts could strongly favor such dissociation by enhancing both $\sigma_c$ and $P_s$.

Fig. 3 illustrates the possible effect of Pt-drugs on a DNA molecule and the potential energy curve resulting in enhanced DEA to the phosphate group, which subsequently leads
Fig. 3. (a) A double helix DNA (I) and the formation of a TNI on the phosphate group in the absence (II) and presence (III) of Pt-drugs. Formation of Pt-adducts such as interstrand crosslinks between two guanines, as shown here, causes to distort DNA conformation by unwinding and bending its double helix. Such distortion modifies physical and chemical stability of the DNA leading to the weakening of the chemical bonds, which could enhance the formation of a TNI and its decay into DEA. X corresponds to NH$_3$, NH$_3$ and C$_6$H$_5$(NH$_2$)$_2$ for cisplatin, carboplatin and oxaliplatin, respectively. dR denotes the deoxyribose moiety. (b) Schematic diagram showing potential energy curves for negatively-charged singly (curve I) and doubly charged phosphate groups (i.e., formation of a TNI) in the absence and presence of Pt-drugs (curves II and III, respectively). $R_E$ is the equilibrium internuclear distances of the singly charged phosphate group. $R_C$ and $R_{C'}$ are the crossing points in unmodified and modified DNA, respectively. The presence of Pt-adducts is expected to shift the crossing point towards $R_E$ (i.e., $R_{C'} < R_C$) resulting in enhanced DEA and subsequently formation of DNA strand breaks by subexcitation-energy electrons. F-C region and AD denote transition in Frank-Condon region and autodetachment, respectively.
to the rupture of phosphodiester bond. Since Pt-adducts reduce the chemical stability of the DNA double helix, resulting in the weakening of the chemical bond at the platination site (11), they could modify the range of nuclear separation over which dissociation takes place.

**CONCLUSION:**

We have reported that the near-zero-eV electrons efficiently induce DSB and SSB in DNA modified by the Pt-drugs cisplatin, carboplatin and oxaliplatin. A comparison of the yields of damage shows that electrons of 0.5 eV are more efficient than 1486-eV X-ray photons and 10-eV electron in the induction of SSB, particularly in the presence of Pt-adducts. In addition, these subexcitation-energy electrons are as efficient as X-rays and electrons of 10 eV for the formation of DSB in DNA modified by Pt-drugs. With respect to the major role of LEEs in the enhancement of damage to modified DNA, the present study shows that subexcitation-energy electrons make a significant contribution to the enhanced damages and hence DEA is the main process responsible for the radiosensitization of DNA by Pt-drugs. In addition, the formation of DSB by such electrons is suggested a single-hit process for DSB, particularly at low doses such those employed in clinics. Carboplatin and oxaliplatin have higher efficiency than cisplatin in the enhanced formation of DSB and SSB by 0.5-eV electrons. For the damages induced by 10-eV electrons or 1.5-keV X-rays, such a higher efficiency has also been observed in the presence of carboplatin, while oxaliplatin has a similar efficiency to cisplatin. The modification of DNA by Pt-drugs is an extremely efficient means of increasing DSB formation induced by subexcitation-energy electrons, which constitute a major product generated by high-energy irradiation in cells.

Alteration in the chemical and physical stability of DNA in the vicinity of Pt-adducts appears to perturb the properties of the target molecule such as band structure, charge polarization and internuclear separations between elements, resulting in the enhancement of the DEA probability via shape resonances by increase in both the probability of electron capture and anion survival. This enhancement of the magnitude of DEA can be explained by referring to Eq. (1), which indicates that a lowering the energy of a TNI due to the high positive electron affinity of Pt should shift the crossing point $R_c$ closer to the equilibrium nuclear separation of the electronic ground state $R_E$. Such a shift reduces considerably the magnitude of the integral in Eq. (1) causing $\sigma_{DEA}$ to increase exponentially. Within DNA,
the reduction of the energy of a TNI also results in a decrease of the number of vibrational
decay channels of the anion state, which increase its lifetime and hence reduces $\Gamma_a$. This
reduction caused by the presence of the Pt atom should also reduce exponentially $P_s$ in Eq.
(1). More generally, the present study may lay the groundwork to effectively improve
radiosensitization by investigating nanoscale quantum processes such as DEA.

**MATERIALS AND METHODS**

**Sample Preparation and Manipulation.** Plasmid DNA (pGEM-3Zf, 3199 base pairs) was extracted from *E.coli* JM109 and purified with a HiSpeed plasmid Maxi kit (QIAGEN). The DNA was then redissolved in TE buffer (10 mM Tris, 1 mM EDTA) with pH 8 to protect the plasmid DNA from degradation. Prior to use, DNA was cleaned up from TE by applying a home-made microcolumn of Sephadex G-50 resin on a bed of glass beads, which is highly efficient for the removal of small molecules, i.e. Tris and EDTA, from a solution. The concentration of DNA and its purity were then determined by measuring the ratio of ultraviolet (UV) absorption of DNA and the contaminating protein at 260 and 280 nm, respectively, with a Synergy HT-I spectrophotometer. For the plasmid DNA used in this study, this ratio was 1.99 which corresponds to purity greater than 95% (45). Analysis of these plasmids by agarose gel electrophoresis showed that the purified plasmid DNA consisted of 96% supercoiled, 2% concatemeric, and 2% nicked circular forms.

Cisplatin, carboplatin and oxaliplatin were purchased from Sigma-Aldrich with stated purities of higher than 98% and used without further purification. Their solutions were prepared in ddH$_2$O based on their molar solubility. Tris buffer was also added to the solution of DNA in ddH$_2$O at a ratio of one tris compound per nucleotide and the final solution was split into four parts. According to the kinetics of binding cisplatin, carboplatin and oxaliplatin to DNA (22), the solution of each Pt-drug was added to one part of the latter DNA solution in the initial concentration ratios of the 20, 40 and 20 cisplatin, carboplatin and oxaliplatin molecules per plasmid DNA, respectively (46). After 45-60 minutes incubation at room temperature in the dark, the unbound Pt-drug molecules, other molecules and complexes with a molecular weight smaller than 1500 g mol$^{-1}$ were then separated from the Pt-drug-DNA solution by gel filtration with a Sephadex G-50 medium.
Such filtration was expected to produce clean solutions of Pt-DNA in ddH2O. The final solution contained only Pt-drug bound to DNA at the average ratio of two Pt-drug molecules per DNA as determined by inductively coupled plasma mass spectroscopy (ICP-MS). Pure DNA samples were also prepared and kept under the same conditions as the Pt-drug-DNA samples.

To prepare the nanoscale films (10 nm thicknesses) of either pure DNA or Pt-drug-DNA, an aliquot of each sample dissolved in ddH2O was spread out on either a clean tantalum (Ta) or a glass surface. The aliquot was frozen (lyophilized) at -65°C for three minutes in a glove box and then dried under a pressure of 1-3 mTorr for about two hours (SI Appendix, Table S2). The samples were then transferred to either one of our electron or X-ray home-made irradiators.

**Sample Irradiation.** For electrons irradiation, the nanoscale film samples were placed on sample holders inside a UHV chamber equipped with a LEE gun. The chamber was evacuated for 24 h by a hydrocarbon-free turbomolecular pump to a pressure of 5 x 10^-9 Torr at room temperature. After stabilization of the electron-beam current at a given current density (9.95 x 10^10 electron s^-1 cm^-2), the DNA films were individually irradiated with electrons of 0.5 eV for various periods between 5 s and 16 min. One of the samples in the UHV chamber was never irradiated with electrons to serve as a control.

For X-rays irradiation, the DNA films were transferred to the X-ray irradiator (SI Appendix, Fig. S1) for exposure to X-rays of varying fluences, in the presence of nitrogen gas having a stated purity of 99.9% and no humidity (30). The 1486 eV Al Kα X-rays were generated from a cold-cathode source. A plasma discharge with a 5.5 mA current is formed between a cathode and an aluminium foil target in a vacuum chamber. Aluminium characteristic Kα X-rays, produced by electron bombardment of the target, travel outside the chamber through a flight tube continuously flushed with helium gas at atmospheric pressure. The X-rays then pass through a thin foil of Mylar to enter a small chamber filled by dry N2 at atmospheric pressure, where plasmid DNA films are deposited on the different substrates. For each group of samples, two lyophilized samples were kept under the same atmospheric experimental conditions as the irradiated samples, but not irradiated by X-rays to serve as controls.
Qualification of DNA Damages. After irradiation, the films were immediately retrieved from the chamber and dissolved within a few minutes in TE buffer from their substrate with 95-98% efficiency. The relative percentage of the different structural forms including supercoiled, nicked circular and linear in each DNA and Pt-drug-DNA sample was obtained by agarose gel electrophoresis. The samples and the agarose gels were stained with SYBR Green I (Molecular Probes, Invitrogen detection technologies) at a concentration of 100X and 10,000X, respectively. The samples were separated on a 1% agarose gel in 1X TAE buffer at 100 volts for 7 minutes followed by 75 volts for 68 minutes (5 V cm⁻¹). The gels were then scanned using a Typhoon-Trio laser scanner (from GE Healthcare) adjusted for the blue fluorescent mode at an excitation wavelength of 488 nm and filter type 520 nm-bandpass (520 BP 40) in the normal sensitivity mode. The amount of each structural form of the DNA was analyzed by ImageQuant (Molecular Dynamics) software.

Calculation of the yields of DNA damages. The yield ($Y'$) for the ultra-LEE- and X-ray-induction of SSB and DSB in DNA, respectively corresponding to the formation of the nicked circular and linear configuration of plasmid in unmodified and Pt-drug modified DNA were derived from linear least-square fits to the initial linear slopes of the respective exposure–response curves. $Y'$ represents the number of the DNA damages per each incident radiation, then they were converted to the yield of damage per absorbed dose (Gy) for each base pair, denoted by $Y$, to compare the efficiency of 0.5-eV electrons and 1486-eV X-rays in the enhancement of the damage formation in the modified DNA.

In the X-ray irradiation, plasmid DNA films were deposited on glass substrate to minimize the formation of secondary electrons from the substrate by the X-rays. For the pGEM-3Zf(−) plasmid DNA, the mass attenuation coefficient ($\mu/\rho$) for 1.5 keV X-rays is $1056 \text{ cm}^2 \text{ g}^{-1}$, based on the atomic composition of DNA and $\mu/\rho$ of individual atoms (31). Thus, for 10-nm thick DNA films, only 0.2% of the soft X-ray photons arriving at the film’s surface interact with DNA, while 99.8% pass through the film to reach the substrates. $Y_{x\text{-ray}}$ is calculated from:

$$Y_{x\text{-ray}} = \frac{Y'_{x\text{-ray}}}{N_{bp}} \left[ \frac{m f_x}{CE_{x\text{-ray}} \left(1 - e^{-\left(\mu/\rho\right) s}\right)} \right].$$ (II-1)
The bracket factor converts the incident energy of X-rays to the absorbed dose in a 10 nm \((h)\) thick solid film composed of 500 ng plasmid DNA \((m)\) with \(\rho = 1.71 \text{ g cm}^{-3}\). \(f_i\) is a correction factor for the kinetic energy carried out of the film by the SEs generated by the incident X-rays inside the films. This factor is 0.67 in our experimental conditions (30).

The yield of damage for 0.5-eV electrons is calculated from:

\[
Y_{0.5\text{eV}} = \frac{Y'_{0.5\text{eV}}}{f_i N_{bp}} \left[ \frac{m}{CE_i \left(1 - e^{-h/\lambda}\right)} \right],
\]

where the \(f_i\) is a correction factor for the effect of film charging on the initial slope of the exposure-response curve for the formation of the DNA damages by LEEs, and \(\lambda\) is the attenuation length of LEEs inducing DNA strand breaks passing through a solid film of plasmid DNA. Assuming all the electrons of incident energy 0.5 eV are absorbed within a 10 nm \((h)\) thick film of 210 ng \((m)\) plasmid DNA, \(f_i\) and \(\lambda\) are 0.29 and 3 nm, respectively (48).

In addition, the ratios between the yields of the modified DNA by Pt-drugs to those of unmodified DNA were determined and referred as an enhancement factor \((EF)\). This factor represents the radiosensitivity index of Pt-drugs in the induction of SSB and DSB by Ultra-LEEs and X-rays.

**ACKNOWLEDGEMENT:**

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REFERENCES


SUPPORTING INFORMATION

S.1. Model for the Radiation Damage to Supercoiled Plasmid DNA

Supercoiled configuration of a plasmid DNA is converted to either circular or linear forms as a result of the formation of SSB or DSB, respectively. A circular form can also be converted to a linear form by the DSB formation. By assuming that radiation induces SSB and DSB via a random process and by ignoring the formation of multiple fragments from a linear DNA due to their negligible amount, SJ McMahon and FJ Currell proposed the following differential equations to describe the response of the plasmid DNA to radiation¹:

\[
\begin{align*}
\frac{dS}{dD} &= -(\beta_s + \beta_d)S, \\
\frac{dC}{dD} &= \beta_s S - \beta_d C - \rho \beta_s^2 D(S + C), \\
\frac{dL}{dD} &= \beta_d (S + C) + \rho \beta_s^2 D(S + C),
\end{align*}
\]

where \( S, C \) and \( L \) are the relative percentage of supercoiled, circular and linear DNA, respectively. The yields of SSB and DSB are proportional to the absorbed dose \( D \) and denoted by \( \beta_s \) and \( \beta_d \), respectively. \( \rho \) is the probability of two SSBs on a single plasmid becoming a DSB that is expressed as the ratio of the maximum distance between two SSBs leading to a DSB divided by the length of plasmid.

In the LEE-irradiation of nanometer-scale solid plasmid DNA films, only a fraction of DNA molecules is exposed to LEEs, due to the limited penetration of the ultrashort-range electrons inside the film ². This effect is observed as a saturation level in the exposure-response curves of the 0.5-eV electron-irradiated films in the Fig. 1. By introducing \( \gamma \) as a fraction of the DNA molecules accessible to LEEs, the analytical integration of Eqs. 1-3 gives:

\[
S(D) = \gamma S_0 e^{-(\beta_s + \beta_d)D} + (1 - \gamma)S_0,
\]
where $S_0$ and $C_0$ are the initial amounts of supercoiled and circular DNA in non-irradiated films. The initial amount of linear DNA is set to zero, since none is detected following gel electrophoresis. For 0.5-eV electron irradiation, the value $\gamma$ is 0.015. In Eq. \((6)\), $\beta_D$ and $\beta_s^2 \rho D/2$ are the rate for the formation of DSB by single- and double-hit processes, respectively. Integrating these rates over radiation dose (i.e., between 0 to $D$) gives the cumulative (total) number of DSB resulted from single- and double-hit processes, $CL(D)_{s-H}$ and $CL(D)_{d-H}$, respectively:

\[
CL(D)_{s-H} = \int_0^D \beta_D dD = \beta_D D 
\]

\[
CL(D)_{d-H} = \int_0^D \frac{1}{2} \beta_s^2 \rho D dD = \frac{1}{4} \beta_s^2 \rho D^2 
\]

Table S1 presents these total numbers of DSB induced by 0.5-eV electrons with the absorbed doses up to 2 Gy, i.e., a typical radiation dose used in clinic, in unmodified DNA and DNA modified by Pt-drugs.

References


Table S1: Total number of DSB ($10^6$) per plasmid induced by 0-2 Gy of 0.5-eV electrons in unmodified DNA and DNA modified by cisplatin, carboplatin or oxaliplatin via single- and double-hit processes.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Double-hit DSB</th>
<th>Single-hit DSB</th>
<th>Ratio of Single- to Double-hit</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0.16</td>
<td>ND</td>
<td>--</td>
</tr>
<tr>
<td>Cisplatin + DNA</td>
<td>0.36</td>
<td>190</td>
<td>530.7</td>
</tr>
<tr>
<td>Carboplatin + DNA</td>
<td>0.74</td>
<td>260</td>
<td>350.6</td>
</tr>
<tr>
<td>Oxaliplatin + DNA</td>
<td>0.43</td>
<td>320</td>
<td>747.7</td>
</tr>
</tbody>
</table>
**Table S2.** Specifications of the DNA films prepared in this study including film thickness $h$ (nm), mass of the DNA $m_{DNA}$ (ng), film area $A$ (cm$^2$). These films were deposited on the either tantalum (Ta) or glass substrates by lyophilisation and irradiated by either electrons or X-rays.

<table>
<thead>
<tr>
<th>Irradiation Condition</th>
<th>$h^*$</th>
<th>$m_{DNA}$</th>
<th>$A$</th>
<th>Substrate**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5-eV electrons†</td>
<td>10</td>
<td>210</td>
<td>0.1256</td>
<td>Ta</td>
</tr>
<tr>
<td>1.486-eV X-ray</td>
<td>10</td>
<td>500</td>
<td>0.385</td>
<td>Glass</td>
</tr>
</tbody>
</table>

* The frozen-dried samples (films) deposited on either tantalum (Ta) or glass had a ring shape of average radius $r$. Assuming a density ($\rho$) 1.71 g.cm$^{-3}$ for the plasmid extracted from *E. coli* (1), the average thicknesses ($h$) of different groups of DNA films were determined by applying the formula: $m_{DNA} = \rho V = \rho Ah = \rho (\pi r^2) h$.

** For electron irradiation, it is important to both monitor the incident beam current during the electron impact and shield the DNA films from the scattered electrons produced during irradiation of other films. Therefore, the metal substrate Ta was chosen to be easily connected to either ground or negative (repulsive) potentials. For soft X-rays experiment, in contrast, an insulator glass substrate was chosen to prevent the generation of secondary electrons from the substrate by X-rays.

† Since 70 ng DNA is required for a precise quantification of various bands in the gel electrophoresis, particularly very small band with contribution to less than 0.5%, 210 ng DNA or Pt-DNA per sample is the minimum amount of DNA to perform the gel electrophoresis three times for each sample. With respect to the sample area, this amount of DNA produces, on average, a 5-monolayer (~ 10 nm) film. Considering the mean free path of 0.5-eV electrons (i.e., a few nanometers), most of the damage to DNA occur in the layers at the topmost part of the film, thereby minimizing the effect of Ta substrate on the interaction of electron with DNA molecules.
Figure S1. Schematic view of the apparatus used to irradiate DNA samples with 1.486 keV Al Kα X-ray photons. The apparatus comprises a chamber evacuated to pressure below 5 mTorr, connected to a pressure gauge (A) and an adjustable leak valve (B) connected to a nitrogen gas source. This valve stabilizes the nitrogen pressure at about 20 mTorr in the main chamber to control the plasma current. A negative potential of 3.4 kV is applied to a concave aluminum cathode (C) through a high-voltage electrical feedthrough (D) fixed in a glass-ceramic (Macor) support (E) and placed as a cap on a long quartz tube (F). A nitrogen plasma discharge with 5.5 mA current is formed between the cathode and an aluminum foil target (G). Aluminum atoms are ionized by electrons incident on the thin foil and characteristic Kα X-rays with energy 1.486 keV are emitted outside the chamber through a flight tube (H) continuously flushed with helium gas at atmospheric pressure. X-ray traverse the helium gas and then a thin foil of Mylar (I) to enter a small chamber, where the plasmid DNA films deposited on the different substrates have been inserted on six aluminum plates of 44.5 mm diameter (J). These
plates are fixed at different positions around a brass rotating disc (K) to allow irradiation of samples directly by X-rays, for different periods of time (i.e., various radiation doses) in the presence of specific amounts of gases or vapours introduced by valves (L). In the present experiments, the distance of $1.7 \pm 0.05$ mm between the Mylar foil and the surface of the plates is occupied by dry N$_2$ at atmospheric pressure. Lyophilized samples of plasmid DNA are placed very close to the Mylar foil to avoid too much photon absorption by the surrounding atmosphere. Furthermore, GAFCHROMIC® HD-810 radiographic dosimetry film (Advanced Materials Group of International Specialty Products Technologies Inc., Wayne, NJ, USA) were used to measure the incident photon fluence for each irradiation period.
II.5. Fifth Article: Cisplatin Enhances the formation of DNA single and double strand breaks by hydrated electrons and hydroxyl radicals.

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Contributions: Mohammad Rezaee designed and performed research, analyzed data and wrote manuscript. Professor Darel Hunting supervised the project, advised on the design and data interpretation, and edited the manuscript. Professor Léon Sanche co-supervised the project and edited the manuscript.
Cisplatin Enhances the Formation of DNA Single and Double Strand Breaks by Hydrated Electrons and Hydroxyl Radicals

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RESULTS – Fifth Article

ABSTRACT
The synergistic interaction of cisplatin with ionizing radiation is the clinical rationale for the treatment of several cancers, including head and neck, cervical and lung. The underlying molecular mechanism of the synergy has not yet been identified, although both DNA damage and repair processes are likely involved. Here, we investigate the indirect effect of γ-rays on strand break formation in a supercoiled plasmid DNA (pGEM-3Zf-) covalently modified by cisplatin. The yields of single and double strand breaks were determined by irradiation of DNA and cisplatin/DNA samples with ⁶⁰Co γ-rays under four different scavenging conditions to examine the involvement of hydrated electrons and hydroxyl radicals in inducing the DNA damage. At 5 mM tris in a N₂ atmosphere, the presence of an average of two cisplatin per plasmid increased the yields of single and double strand breaks by factors of 1.9 and 2.2, respectively, relative to the irradiated unmodified DNA samples. Given that each plasmid of 3200 base pairs contained an average of 2 cisplatin, this represents an increase in radiosensitivity of 3200 fold on a per base pair basis. When hydrated electrons were scavenged by saturating the samples with N₂O, these enhancement factors decreased to 1.5 and 1.2, respectively for single and double strand breaks. When hydroxyl radicals were scavenged using 200 mM tris, the respective enhancement factors were 1.2 and 1.6 for single and double strand breaks, respectively. Furthermore, no enhancement in DNA damage by cisplatin was observed after scavenging both hydroxyl radicals and hydrated electrons. These findings show that hydrated electrons can induce both single and double strand breaks in the platinated DNA but not in unmodified DNA. In addition, cisplatin modification is clearly an extremely efficient means of increasing both single and double strand breaks formation by the hydrated electrons and hydroxyl radicals created by ionizing radiation.

INTRODUCTION
Cisplatin is the first platinum-based chemotherapeutic drug to be administered routinely in a standard chemoradiation therapy procedure (1-6). This molecule enters cells via both passive diffusion and active transporters and subsequently converts to chemically reactive forms including mono- and diaqua-cisplatin due to hydrolysis (7, 8). The activated
cisplatin reacts with cellular components through ligand exchange at the platinum atom (9, 10). Owing to the slow kinetics of the hydrolysis reaction ($t_{1/2} \approx 4$ h), cisplatin can diffuse through the cytoplasm, enter the nucleus and react with DNA (11). It specifically binds to the nitrogen atom at position seven of the purine bases, particularly guanine, to form various DNA adducts including intrastrand cross-links [1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpNpG)], interstrand cross-links (ICL), and monofunctional adducts with guanine (12, 13). The adducts distort the conformation of DNA by unwinding, bending and destabilization of its double helix (11, 13, 14). Such structural alterations are believed to be responsible for radiosensitization effect of cisplatin (15).

Although preclinical and clinical studies have shown that the combination of the drug with radiation increases tumour cell killing and enhances patient survival (4, 16-19), the mechanisms underlying its interaction with radiation have not yet been elucidated. It is suggested that cisplatin adducts can inhibit DNA repair of radiation-induced lesions and/or increase radiation damage to DNA (20-23). Treatment of cultured cells with cisplatin for a period of a few hours up to one day before irradiation leads to reduced repair of DNA sublethal and potentially lethal damage through modification of repair processes, in particular those are responsible for DNA double strand break (DSB) repair (24-27). The effect on repair was observed when the cisplatin-DNA adducts were present in the vicinity of the DNA damage (28). Additionally, the presence of cisplatin in DNA is suggested to result in the preferential formation of radiation-induced DNA damage in the vicinity of the cisplatin-DNA adducts (29).

The biological impact of ionizing radiation results predominantly from the formation of a variety of lesions in cellular DNA via energy deposition into the DNA itself (direct effect) and its surrounding molecular environment, particularly water molecules (indirect effect) (30, 31). Owing to the considerable amount of water in a cell (about 70% of cellular mass) and the high reactivity towards DNA of species arising from water radiolysis, most importantly hydroxyl radicals ($'OH$), hydrated electrons ($e^-_{aq}$) and the atomic hydrogen ($'H$), it is estimated that at least 50% of DNA damage from $\gamma$-irradiation results from the indirect effect due to the water radiolysis (32, 33). $'OH$ is the most reactive radical generated from the radiolysis of water and it interacts with DNA constituents at a rate which is essentially diffusion-controlled (34). This radical induces DNA strand breaks.
via interaction with sugar moieties in the presence or absence of oxygen (35). In contrast, \(^3\)H and \(e^-_{aq}\) are far less important in the production of DNA damage, particularly strand breaks, despite the fact that they are highly reactive reducing species (36). The observation of different ionization potentials for \(e^-_{aq}\) due to the localization of the electron either on the surface or inside a water complex also suggests that the previous notion of \(e^-_{aq}\) as a single equilibrated species in water is too simplistic (37-40). However, experimental and theoretical studies show that \(e^-_{aq}\) cannot induce strand breaks in an aqueous solution of double stranded DNA (36, 41).

In the present study, we have determined the effect of cisplatin on the sensitisation of a supercoiled plasmid DNA toward the indirect effect of \(\gamma\)-rays by measuring the yields of single strand break (SSB) and DSB. The contributions of the most important reactive species in the induction of DNA strand breaks in unmodified and platinated DNA are also estimated in a deoxygenated atmosphere by applying four different scavenging conditions.

**EXPERIMENTAL**

The experimental details of sample preparation, irradiation, and post-irradiation analysis developed for the present studies have been reported elsewhere (42, 43). Here we provide a brief description.  

**Sample Preparation:** Plasmid DNA [pGEM-3Zf(-), 3199 base pairs] was extracted from *E.coli* JM109 and purified with a HiSpeed plasmid Maxi kit (QIAGEN). The purified plasmid DNA consisted of 96% supercoiled, 2% concatemeric, and 2% nicked circular forms. The ratio of ultraviolet absorption of DNA and any contaminating protein at 260 nm and 280 nm, respectively, was 1.99 which corresponds to a purity greater than 95% (44). The TE buffer (Tris-EDTA: 10 mM-1 mM) was separated from DNA by gel filtration with a Sephadex G-50 medium. Thus the final solution consisted of DNA and distilled deionized (dd) H\(_2\)O.

Cisplatin was purchased from Sigma-Aldrich with a stated purity of 99.9% and used without further purification. A solution was prepared in ddH\(_2\)O based on its molar solubility (0.77 x 10\(^{-2}\) after 24 h).
DNA Platination: Tris buffer was added to the DNA solution at a ratio of one tris compound per nucleotide. According to the kinetics of binding of cisplatin to DNA, the cisplatin solution was added to the DNA solution at a ratio of 20 cisplatin molecules per plasmid DNA (≈ 0.003 cisplatin molecules per nucleotide). After 45 minutes incubation at room temperature, the unbound cisplatin molecules, tris compounds, and complexes of tris with cisplatin were then separated from the cisplatin/DNA solution by gel filtration with a Sephadex G-50 medium. The final solution consisted of cisplatin bound to DNA at an average ratio of two cisplatin molecules per DNA (~ 0.0003 cisplatin molecules per nucleotide) as determined by inductively coupled plasma mass spectroscopy (ICP-MS). A pure DNA sample was also prepared and kept under the same conditions as the cisplatin/DNA sample.

Deoxygenation and Radical Scavengers: Tris buffer as a *OH scavenger was added to the solutions of cisplatin/DNA and DNA to obtain final concentrations of either 5 or 200 mM corresponding to scavenging capacities of 7 x 10^6 and 3 x 10^8 s^-1, respectively (45), while the reaction rate of *OH with DNA is 2.5 x 10^8 s^-1 (35). The final samples were prepared in 20 μl solutions containing 500 ng of either DNA or cisplatin/DNA at a concentration of 25 ng/μl. Deoxygenation of the samples was performed by bubbling the solutions with either N₂ or N₂O before irradiation. N₂O was also used as a scavenger of e⁻o₂.

Irradiation: Cisplatin/DNA and DNA samples in sealed tubes were irradiated using a ⁶⁰Co-GammaCell 200 (Atomic Energy of Canada) emitting γ-rays with energies of 1.18 and 1.33 MeV and a dose rate of 1.4 Gy/min at various doses. The irradiation was carried out at the room temperature immediately after saturation of the samples with N₂ and N₂O.

Quantification of DNA Strand Breaks: After irradiation, the percentage of the different structural forms including supercoiled, nicked circular and linear in each DNA and Pt-DNA sample was obtained by agarose gel electrophoresis. The samples and the agarose gels were stained with SYBR Green I (Molecular Probes, Invitrogen detection technologies) at a concentration of 100X and 10,000X, respectively. Previous studies show that SYBR Green I is less dependent on the length of the DNA and more sensitive than other nucleic acid fluorescent dyes such as ethidium bromide (46). It has also been shown that the fluorescent intensity of SYBR Green I bound to DNA increases linearly as a function of DNA concentration over three orders of magnitude (47). The samples were separated on a 1%
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agarose gel in 1X TAE buffer at 100 volts for 7 minutes followed by 75 volts for 68 minutes (5 V cm⁻¹). The gels were then scanned using a Typhoon-Trio laser scanner (from GE Healthcare) adjusted for the blue fluorescent mode at an excitation wavelength of 488 nm and filter type 520 nm-bandpass (520 BP 40) in the normal sensitivity mode. The amount of each structural form of the DNA was analyzed by ImageQuant (Molecular Dynamics) software. To achieve better accuracy, the binding efficiencies of SYBR Green I for the same amount (75 ng) of supercoiled and linear DNA were measured to establish a correction factor. This factor, which arises from the weaker binding of SYBR Green I to supercoiled DNA than to the nicked circular and linear forms, was 1.4 and applied to the quantification of the different structural forms of plasmid DNA.

**Determination of the Yields of SSB and DSB:** The yield for the induction of DNA SSB and DSB corresponding to the formation of the nicked circular and linear forms, respectively, by γ-rays in both types of the samples were derived from the initial linear slopes (S in percentage of SSB or DSB per Gy) of the respective dose–response curves:

\[ Y = \frac{S}{f_n} \]

where \( f_n \) is the normalization factor. For the yields of SSB and DSB, the factor has values equal to the percentage of the supercoiled DNA in the non-irradiated sample and 100, respectively.

In addition, the ratios between the yields of either SSB or DSB in the presence of cisplatin (\( Y_{cis\,DNA} \)) to those obtained in the absence of cisplatin (\( Y_{DNA} \)) were determined and referred as Enhancement Factor (EF):

\[ EF = \frac{Y_{cis\,DNA}}{Y_{DNA}} \]

EF represents the radiosensitivity effect of cisplatin in the induction of SSB and DSB by ionizing radiation.

**RESULTS AND DISCUSSION**

DNA strand breaks induced by \( e^-_{aq} \) and \( {^\cdot}OH \) generated by γ-irradiation:
Fig. 1 presents dose-response curves for the formation of nicked circular and linear DNA in the DNA and cisplatin/DNA samples irradiated with γ-rays in 5 mM tris in the presence of either N$_2$O, a scavenger of $e_{aq}^-$, or N$_2$. The N$_2$ is used to displace O$_2$, which scavenges $e_{aq}^-$ from the solution. The platinated plasmid contained an average of two cisplatin per plasmid (i.e., 1 platinum per 1600 bp). With this ratio and assuming a Poisson distribution for the cisplatin among the plasmids, about 13% of the plasmids should have no cisplatin. Similar ratios of cisplatin to DNA have been observed in clinical applications, particularly in the malignant tissues of the patients treated with liposomal cisplatin (48). Table 1 presents the yields of SSB and DSB induced by γ-rays in DNA and cisplatin/DNA samples as well as the enhancement factors for the formation of the strand breaks when cisplatin is bound to the DNA. The presence of cisplatin increases both SSB and DSB formation by approximately 2 fold in the γ-irradiated plasmid. Given that each plasmid (of 3200 bp) contains an average of 2 cisplatin adducts, this represents an increase in radiosensitivity of about 3200 fold on a per base pair basis, assuming the cisplatin only sensitizes the two bases to what it is bound. In a dilute aqueous solution of DNA containing 5 mM tris under N$_2$, all radiolysis species, $^\cdot$OH, $^\cdot$H and $e_{aq}^-$ are free to react with DNA. Under these conditions, dose-response curves for the formation of SSB and DSB (panels A and C in fig. 1, respectively) show that the levels of damage in cisplatin/DNA substantially increase relative to DNA samples. Comparison of the yields (Table 1) also shows that γ-rays enhance the induction of SSB and DSB by factors of 1.9 and 2.2 in the presence of cisplatin, respectively. Since previous experimental and theoretical studies suggest that $e_{aq}^-$ can not induce strand breaks in unmodified DNA (49, 50), one would expect the elimination of $e_{aq}^-$ to have no effect on strand break formation. However, by saturating the DNA solution with N$_2$O, $e_{aq}^-$ reacts with N$_2$O and generates $^\cdot$OH based on the reaction (35):

$$e_{aq}^- + N_2O \rightarrow N_2 + OH^-$$

Under these conditions, as shown in panels B and D in Fig. 1, strand break formation is
Fig. 1. Dose-response curves for the formation of circular and linear DNA by $^{60}$Co γ-rays for the DNA and cisplatin DNA samples in 5 mM tris. Panels A and B indicate the curves for the circular DNA in the samples saturated with N$_2$ and N$_2$O, respectively. The curves for the linear DNA are shown in the panels C and D for the samples saturated with N$_2$ and N$_2$O, respectively. Data in A–D are means ± SD from three experiments. They have been fitted by employing a least squares regression analysis.
greater than that observed with a N\textsubscript{2} atmosphere due to the additional 'OH produced from scavenging $e^-\text{aq}$. Since the yield of $e^-\text{aq}$ by radiolysis is the same as for 'OH (2.8 x 10\textsuperscript{-7} mol J\textsuperscript{-1}) in a dilute aqueous solution, the complete scavenging of $e^-\text{aq}$ should double the yield of 'OH (50). Thus, for the irradiated unmodified DNA, this expectedly leads to an approximately 2-fold increase in the yields of both SSB (6.4 x 10\textsuperscript{-7} to 12.6 x 10\textsuperscript{-7} SSB/Gy/bp) and DSB (1.8 x 10\textsuperscript{8} to 3.4 x 10\textsuperscript{8} DSB/Gy/bp). These results are consistent with the substantial role of 'OH in the induction of strand breaks in unmodified DNA. However, if $e^-\text{aq}$ were as efficient as 'OH at creating strand breaks in unmodified DNA, the conversion of $e^-\text{aq}$ to 'OH by N\textsubscript{2}O addition should have no effect on strand break yields. In the presence of cisplatin, the yield of SSB under N\textsubscript{2}O increases by a factor of 1.6 (12 x 10\textsuperscript{-7} to 19 x 10\textsuperscript{-7} SSB/Gy/bp) but interestingly, no increase is observed in the yield of DSB in the N\textsubscript{2}O atmosphere (4 x 10\textsuperscript{8} DSB/Gy/bp). Furthermore, the cisplatin enhancement factors for SSB and DSB are 1.5 and 1.2 in the presence of N\textsubscript{2}O, respectively, which are smaller than those observed under the N\textsubscript{2} atmosphere. These results suggest that $e^-\text{aq}$ must make an important contribution (estimated to be ~ 48 and 20%, respectively) to the formation of DSB and SSB in the cisplatin-modified DNA but not in unmodified DNA.

Table 1. Yields of SSB and DSB (Strand Break/Gy/base pair) induced by the $^{60}$Co y-rays in DNA and cisplatin/DNA samples containing 5 mM tris and saturated with either N\textsubscript{2} or N\textsubscript{2}O. Enhancement factors present increase in the yields due to the presence of cisplatin.

<table>
<thead>
<tr>
<th>DNA Damage*</th>
<th>Atmosphere</th>
<th>Pure DNA</th>
<th>Cisplatin/DNA</th>
<th>Enhancement Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSB</td>
<td>N\textsubscript{2}</td>
<td>(6.4 ± 0.9) x 10\textsuperscript{-7}</td>
<td>(12.0 ± 1.0) x 10\textsuperscript{-7}</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>SSB</td>
<td>N\textsubscript{2}O</td>
<td>(12.6 ± 1.1) x 10\textsuperscript{-7}</td>
<td>(19.0 ± 1.6) x 10\textsuperscript{-7}</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>DSB</td>
<td>N\textsubscript{2}</td>
<td>(1.8 ± 0.1) x 10\textsuperscript{8}</td>
<td>(4.0 ± 0.1) x 10\textsuperscript{8}</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>DSB</td>
<td>N\textsubscript{2}O</td>
<td>(3.4 ± 0.2) x 10\textsuperscript{8}</td>
<td>(4.0 ± 0.3) x 10\textsuperscript{8}</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

* SSB: Single Strand Break; DSB: Double Strand Break
Fig. 2 presents dose-response curves for the formation of nicked circular and linear DNA in the DNA and cisplatin/DNA samples irradiated with γ-rays in 200 mM tris, an efficient ·OH scavenger, in the presence of either N₂ or N₂O. Under these conditions (i.e., a ·OH scavenging capacity of 3 x 10⁸ s⁻¹, equal to the ·OH diffusion distance of 6 nm, a situation similar to the cell condition (51)) which minimize ·OH-mediated DNA strand breaks, it is possible to directly examine the contribution of ·OH to the formation of SSB and DSB in the presence and absence of cisplatin. However, the reaction of ·OH with tris compounds induces tris radicals that can subsequently interact with DNA. In contrast to the secondary radicals derived from other scavengers such as DMSO and t-butyl alcohols that result in substantial increases in the yields of SSB under anoxic conditions (52), tris radicals have been shown to be ineffective in the production of DNA strand breaks (43).

Table 2 presents the yields of SSB and DSB induced by the γ-rays in the DNA and cisplatin/DNA samples as well as the enhancement factors for the strand break formation when cisplatin is bound to DNA at an average ratio of 2 cisplatin per plasmid. The formation of SSB and DSB greatly decrease for both DNA and cisplatin/DNA when ·OH is scavenged (panels A and C in Fig. 2). Under N₂ and in the absence of cisplatin, the high scavenging capacity results in an about 2.5-fold decrease in the yields of SSB (6.4 x 10⁻⁷ versus 2.5 x 10⁻⁷ SSB/Gy/bp) and DSB (1.8 x 10⁻⁸ versus 0.8 x 10⁻⁸ DSB/Gy/bp). Such decreases, when ·OH is scavenged, have also been reported in previous studies (43, 53-57).

In DNA modified by cisplatin, the effect of scavenging ·OH is greater, generating 4- and 3-fold reductions in the yields of SSB (12.0 x 10⁻⁷ versus 3.0 x 10⁻⁷ SSB/Gy/bp) and DSB (4.0 x 10⁻⁸ versus 1.3 x 10⁻⁸ DSB/Gy/bp), respectively. As shown in the dose-response curves (panels A and C in Fig. 2), these larger reductions in the yields results in a level of strand breaks in the modified DNA which is only slightly greater than that in the unmodified DNA. Additionally, comparison of the yields measured under these conditions shows small enhancements due to cisplatin, by factors of 1.2 and 1.6 in SSB and DSB, respectively (Table 2). These findings demonstrate the contribution of ·OH in the enhancement of SSB and DSB in the presence of cisplatin.
Fig. 2. Dose-response curves for the formation of circular and linear DNA by $^{60}$Co γ-rays for the DNA and cisplatin DNA samples in 200 mM tris. Panels A and B indicate the curves for the circular DNA of the samples saturated with N$_2$ and N$_2$O, respectively. The curves for the linear DNA are shown in the panels C and D for the samples saturated with N$_2$ and N$_2$O, respectively. Data in A – D are means ± SD from three experiments. They have been fitted by employing a least squares regression analysis.
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Saturating the samples containing 200 mM tris with N$_2$O allows a direct evaluation of the effect of e$_{aq}^-$ on DNA strand break formation. In the absence of cisplatin, the yields of both SSB and DSB increase very slightly presumably because the additional *OH generated by the reaction of N$_2$O with e$_{aq}^-$ are mostly quenched by the 200 mM tris. In the cisplatin-modified DNA, the yields of SSB and DSB are reduced and as a result, the levels of the strand break formation, as shown in dose-response curves in Fig. 2 (panels B and D), become similar in both DNA and cisplatin/DNA samples. These results confirm the substantial role of e$_{aq}^-$ in the induction of SSB and DSB in the presence of cisplatin. Thus, when both *OH and e$_{aq}^-$ are efficiently scavenged, no significant enhancement in the SSB and DSB formation is observed in the modified DNA. These findings suggest that *OH and e$_{aq}^-$ are the main water radiolysis species which synergistically interact with cisplatin to enhance DNA SSB and DSB.

**Contribution of water radiolysis species to DNA strand break formation:**

The radiolysis yields of water radicals have previously been determined for experimental conditions similar to those in the present study (50, 58). It should also be

Table 2. Yields of SSB and DSB (Strand Break/Gy/base pair) induced by the $^{60}$Co $\gamma$-rays in DNA and cisplatin/DNA samples containing 200 mM tris and saturated with either N$_2$ or N$_2$O. Enhancement factors present increase in the yields due to the presence of cisplatin.

<table>
<thead>
<tr>
<th>DNA Damage*</th>
<th>Atmosphere</th>
<th>Pure DNA</th>
<th>Cisplatin/DNA</th>
<th>Enhancement Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSB</td>
<td>N$_2$</td>
<td>(2.5 ± 0.1) x 10$^{-7}$</td>
<td>(3.0 ± 0.2) x 10$^{-7}$</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>SSB</td>
<td>N$_2$O</td>
<td>(2.8 ± 0.2) x 10$^{-7}$</td>
<td>(2.8 ± 0.3) x 10$^{-7}$</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>DSB</td>
<td>N$_2$</td>
<td>(0.8 ± 0.1) x 10$^{-8}$</td>
<td>(1.3 ± 0.1) x 10$^{-8}$</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>DSB</td>
<td>N$_2$O</td>
<td>(1.1 ± 0.1) x 10$^{-8}$</td>
<td>(1.2 ± 0.1) x 10$^{-8}$</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

* SSB: Single Strand Break; DSB: Double Strand Break
noted that the direct effect of ionizing radiation has a negligible contribution to DNA damage in our study since more than 99% of the radiation energy should be absorbed by the water of our dilute aqueous solutions of DNA (35). Since $e^-_{aq}$ does not produce strand breaks in unmodified DNA, strand breaks in the presence of N$_2$ at 5 mM tris must result from $^\cdot$OH and $^\cdot$H. Therefore, total yield of SSB, $Y_{SSB}$, is the sum of the SSB yields induced by $^\cdot$OH and $^\cdot$H:

$$N_2 \text{ Atmosphere} \quad Y_{SSB} = Y_{^\cdot OH} + Y_{^\cdot H}. \quad (3)$$

where $Y_{^\cdot OH}$ and $Y_{^\cdot H}$ are the yields of SSB produced by $^\cdot$OH and $^\cdot$H radicals, respectively.

In the presence of N$_2$O, the yield of $^\cdot$OH should double if quenching is complete, whereas the yield of $^\cdot$H will be the same as in an N$_2$ atmosphere. Thus $Y_{SSB}$ in the presence of N$_2$O is:

$$N_2O \text{ Atmosphere:} \quad Y_{SSB} = 2Y_{^\cdot OH} + Y_{^\cdot H}. \quad (4)$$

In the absence of cisplatin, the measured values of $Y_{SSB}$ under N$_2$ and N$_2$O are 6.4 x 10$^{-7}$ and 12.6 x 10$^{-7}$ SSB/Gy/bp, respectively. Thus, $Y_{^\cdot OH}$ and $Y_{^\cdot H}$ are simply obtained from Eq. (3) and (4). They have values of 6.2 x 10$^{-7}$ and 0.2 x 10$^{-7}$ SSB/Gy/bp, respectively. Accordingly, the contribution of $^\cdot$H to the induction of SSB is about 3% that of $^\cdot$OH, in agreement with other studies (55, 59). These results again confirm the well-established role of $^\cdot$OH in producing DNA strand breaks in unmodified DNA.

In contrast, in the presence of cisplatin, our results show that both $e^-_{aq}$ and $^\cdot$OH contribute to the induction of SSB and DSB leading to the considerable enhancements in DNA damage compared to unmodified DNA. The radiolysis yield of $^\cdot$H is about 10-20% of that of $^\cdot$OH and $e^-_{aq}$. In addition, it has a negligible contribution to the induction of strand breaks. Thus, its contribution to SSB and DSB formation in cisplatin modified DNA is assumed to be the same as in the unmodified DNA (0.2 x 10$^{-7}$ SSB/Gy/bp and 0.2 x 10$^{-8}$ DSB/Gy/bp). In principle, hydrogen peroxide (H$_2$O$_2$) can also mediate DNA damage due to the reaction with the platinum atom of cisplatin leading to the generation of additional $^\cdot$OH based on the Fenton-type reaction (60). However, it will have a negligible contribution to
strand break formation in our study due to the small concentration of cisplatin (25 nM) in the cisplatin/DNA solutions. Therefore, the $Y_{SSB}$ under N$_2$ and N$_2$O atmospheres and in the presence of cisplatin are:

$$Y_{SSB} = Y_{\cdot OH} + Y_e + Y_H$$ (5)

$$Y_{SSB} = 2Y_{\cdot OH} + Y_H$$ (6)

where $Y_e$ is the yield of SSB induced by $e^-_{aq}$. By measuring $Y_{SSB}$ under N$_2$ and N$_2$O, and knowing $Y_H$, $Y_{\cdot OH}$ and $Y_e$ are determined from Eq. (5) and (6). Similarly, such equations allow us to calculate the contribution of each radiolysis species to the induction of DSB.

Table 3 presents the yields of $\cdot OH$, $\cdot H$ and $e^-_{aq}$ in the formation of SSB and DSB in the DNA and cisplatin/DNA samples in 5 mM tris under a N$_2$ atmosphere. It is observed that in the presence of cisplatin, $\cdot OH$ enhances slightly the yields of SSB and DSB by factors of 1.5 and 1.2, respectively. Despite such increases, the relative contribution of $\cdot OH$ to DNA strand break formation, particularly for DSB, is actually smaller in the platinated DNA than that in the unmodified DNA. In the absence of cisplatin, $\cdot OH$ contributes to the induction of at least 97% and 90% of total SSB and DSB, respectively, whereas the contributions decline to 78 and 48% in the presence of cisplatin, because of the contribution of $e^-_{aq}$ which induces 20% and 48% of total SSB and DSB, respectively, in cisplatin/DNA samples, as

<table>
<thead>
<tr>
<th>Radiolysis Species</th>
<th>Yield of SSB (x10$^{-7}$ SSB/Gy/bp)</th>
<th>Yield of DSB (x10$^{-8}$ DSB/Gy/bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>Cisplatin/DNA</td>
</tr>
<tr>
<td>$\cdot OH$</td>
<td>6.2 ± 0.8</td>
<td>9.4 ± 0.9</td>
</tr>
<tr>
<td>$\cdot H$</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>$e^-_{aq}$</td>
<td>--</td>
<td>2.4 ± 0.4</td>
</tr>
</tbody>
</table>
opposed to the unmodified DNA where no strand breaks are produced by $e_{aq}^-$. 

**Reaction of the radiolysis species with the cisplatin-DNA:**

The binding of cisplatin to supercoiled DNA is known to alter the conformation of the DNA causing the double helix to bend and unwind in the vicinity of the platination site (12, 61-63). Such structural changes have been observed for all cisplatin-DNA adducts, but each adduct distorts the DNA structure in a distinctive manner. Intrastrand cross-links, in particular 1,2-d(GpG) bend the DNA duplex toward the major groove, thus unwind the DNA and result in a wider and more shallow minor groove (11). Since the deoxyribose moieties are normally on the surface of the minor groove in B-form DNA (64), this unwinding makes the sugar moieties more accessible to the water radicals, especially 'OH. This increased accessibility in combination with the high reactivity of this radical towards the sugar moieties leads to the DNA strand breaks, in particular via H-abstraction from C(4') in the absence of oxygen (35, 60). This may explain the observed enhancement in DNA strand break formation by 'OH in the platinated DNA. On the other hand, ICL formed by cisplatin bend the DNA toward the minor groove leading to a widening of the major groove (65). Studies on the platination of supercoiled DNA also suggest that at a low concentration of cisplatin (less than 1 cisplatin molecule per 1000 bp), the number of ICL is enhanced in the supercoiled form relative to the relaxed and linear DNA forms (66, 67). Considering the average ratio of cisplatin to the plasmid DNA used in the present study (1 cisplatin molecule per 1600 bp, approximately), it is likely that ICL would be the major adducts in our experiments. The structural changes due to ICL result in a higher accessibility of the DNA bases and the cisplatin molecule to the water radiolysis species, particularly $e_{aq}^-$. 

The $e_{aq}^-$ can react with many compounds, including DNA bases, via the process of dissociative electron transfer. The process has two steps: electron attachment to a target molecule and electron transfer to a specific bond causing cleavage of the bond. Attachment of a $e_{aq}^-$ to a molecule depends on the vertical binding energy (VBE) of the electron and adiabatic electron affinity (AEA) of the molecule. The latter associates with the potential energy curves of the neutral and anionic molecules (68) and the former is the energy
required to separate the electron from the water, more specifically from its hydration cavity in the cluster anion, with no molecular rearrangement (37, 38). Thus VBE depends on the size \( n \) of the anionic water cluster \([\text{(H}_2\text{O)}_n^-]\) containing an excess electron. The presence of polar molecules such as water and \( \text{NH}_3 \) in the neighborhood of the target molecule has also been reported to significantly increase the ability of the molecule to capture electrons with energies near zero and even less than zero electron-volts due to the modification in the potential energy curves of neutral and the respective anionic molecules (68-70). It has been suggested that such a modification of AEA at the surface between the DNA molecule and the surrounding water medium would result in AEA in the range \(-1\) to \(-2\) eV that might permit the attachment of \( e^-_{\text{aq}} \) to the solvated bases (40, 71-73). The presence of cisplatin in the solvated DNA could further enhance the probability of electron capture due to: 1- the electron affinity of cisplatin, 2- any structural modification of DNA leading to a higher accessibility of the DNA constituents and cisplatin to polar molecules such as water and to radiolysis species, 3- the presence of two \( \text{NH}_3 \) groups in cisplatin molecules due to their polarity. Therefore, cisplatin should favour the attachment of the \( e^-_{\text{aq}} \) to the solvated DNA, particularly to the bases and cisplatin molecule. Theoretical studies on the solvated DNA show that an electron captured by guanine tends to transfer to the backbone of the DNA and form a phosphate-centered radical anion (68). In the unmodified DNA, additionally, theoretical studies have calculated that the energy barrier required for dissociation of the phosphate group leading to strand break formation is about 30 kcal/mol (41), and hence the \( e^-_{\text{aq}} \) seems unlikely to be able to induce strand breaks in an aqueous solution of DNA (i.e., a medium with a high dielectric constant). However, the structural and chemical modifications observed in the modified DNA (12), particularly in the vicinity of the cisplatin may reduce the energy barrier and favour bond dissociation leading to the formation of strand break.

**CONCLUSION**

We report the effects of cisplatin on the sensitization of supercoiled plasmid DNA toward water radiolysis species. It is shown that the yields of DNA strand breaks substantially increase in the presence of cisplatin. The present results demonstrate that both
'OH and $e^-_{aq}$ increase the formation of SSB and DSB in DNA containing very low levels of cisplatin adducts. The estimated contributions of the radiolysis species inducing the strand breaks show that $e^-_{aq}$ plays an important role in radiation-induced DSB in cisplatin/DNA samples. When both 'OH and $e^-_{aq}$ were scavenged, the yields of the strand breaks were unaffected by the presence of cisplatin, suggesting that the main species responsible for the enhanced damages are 'OH and $e^-_{aq}$.

It is well known that cisplatin physically and chemically affects the DNA molecule, particularly at the site of platination. It is therefore proposed that the structural alterations in the modified DNA increase the accessibility of the radiolysis species to DNA constituents and the chemical changes modify the reactions between the species with the DNA.

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III. DISCUSSION

III.1 Pt-drugs enhance the formation of DNA damage by both the direct and indirect effects of radiation.

Our results indicate that the both direct and indirect effects of ionizing radiations contribute to the enhanced formation of damage in DNA modified by Pt-drugs. The indirect effect of radiation increases different types of damage, including SSB, DSB and interduplex CL to modified DNA, while the direct effect mainly enhances the formation of cluster damages (i.e., DSB and interduplex CL) in the platinated DNA.

In the direct effect, we showed in articles 3 that the yields of damage and the $EF$s for modified DNA irradiated by 10-eV electrons are larger than those by 10-keV electrons. These electron irradiations were performed for the nanometer-scale frozen-dried DNA films deposited on tantalum substrate under ultra-high vacuum condition. The yields of DSB formation by 10-eV electrons, for example, increase by a factor of 3 relative to those by 10-keV electrons for the DNA modified by cisplatin and carboplatin. Since interaction of 10-keV electrons with DNA generates a variety of secondary species including radicals, ions and LEEs, it is inferred from the comparison of the damage yields and $EF$s that LEEs are the main secondary species produced by high-energy ionizing radiation responsible for the observed enhancement of DNA damage in the presence of Pt-drugs. At 10 eV, electrons predominantly induce damage to DNA via two main mechanisms, including $DEA$ and dissociative electronic excitation. While $DEA$ is a decay channel for a TNI formed in either ground or excited states, dissociative electronic excitation can only occur for a TNI decaying into an excited state. Since the minimum required energy for the excitation of organic molecules is about 4-5 eV, the damage to DNA by subexcitation-energy electrons must necessarily occur by $DEA$. In article 4, we showed that 0.5-eV electrons induce DSB in DNA modified by Pt-drugs, essentially via $DEA$, but not in unmodified DNA. In the presence of Pt-DNA adducts, the yields of DSB formation by 0.5-eV electrons are similar to those by 10-eV electrons. For the formation of SSB by 0.5-eV electrons, the respective yields are greater than those by 10 eV
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electrons. These findings indicate that DEA is the main mechanism responsible for the enhanced damage to DNA modified by Pt-drugs induced by sub-excitation electrons.

In the indirect effect of radiation, we demonstrated in article 5 that both 'OH and \( e_{aq}^- \) enhance the formation of SSB and DSB in DNA modified by cisplatin. 'OH contributes 57% and 14% to the enhanced formation of SSB and DSB, respectively. For \( e_{aq}^- \), the corresponding contributions are 43% and 86%, respectively. In contrast to modified DNA, \( e_{aq}^- \) are not able to induce any strand breaks in unmodified DNA. Our finding indicates that 'OH mostly enhances the formation of SSB, while \( e_{aq}^- \) are the main radiolysis species to induce DSB in the platinated DNA. The probability of strand breaks induced by 'OH depends on the accessibility of the sugar moiety, particularly C4' to the radical attack (Clemens von Sonntag, 2006; Sy, Savoye, Begusova, Michalik, Charlier and Spotheim-Maurizot, 1997). The size of a minor groove in B-formed DNA substantially influences the accessibility to C4', which is drastically diminished when the width of the minor groove is less than 4.5 Å (Clemens von Sonntag, 2006). Structural distortion of DNA double helix by Pt-drugs could increase such accessibility. In the presence of 1,2-intrastrand d(GpG) cisplatin adduct, for example, the minor groove width of the DNA measured by X-ray crystallography and NMR spectroscopy has a range between 5.5 – 12.5 Å (Dunham, Dunham, Turner and Lippard, 1998; Gelasco and Lippard, 1998; Ohndorf, Rould, He, Pabo and Lippard, 1999; Takahara, Rosenzweig, Frederick and Lippard, 1995; Takahara, Frederick and Lippard, 1996). Therefore, distortion of DNA conformation by Pt-drugs could play an important role in the enhanced damage to DNA by 'OH. In addition to such accessibility, theoretical and experimental studies have shown that a weakly-bound electron in a water complex can transfer to cisplatin due to the high polarity of its NH₃ groups and form a TNI which may subsequently dissociate the molecule via DEA (Kuduk-Jaworska, Chojnacki and Jański, 2011; Lu, Kalantari and Wang, 2007). Such a process could be responsible for the formation of SSB and DSB by \( e_{aq}^- \) in modified DNA. With respect to the substantial contribution of \( e_{aq}^- \) to the enhanced damage to platinated DNA, DEA can also be
Fig. III.1. Enhancement factors in the yields of SSB, DSB and interduplex CL induced by 10-eV electrons in solid films of DNA and $^{60}$Co gamma-rays in the aqueous solution of DNA in the presence of cisplatin.

considered as the main mechanism for the indirect effects of radiation on DNA modified by Pt-drugs.

The histograms in Fig III.1 show the $EF$s for the formation of SSB, DSB and interduplex CL by $\gamma$-rays and electrons of 10 eV in modified DNA at the average ratio of two Pt-DNA adducts per plasmid. The $\gamma$-rays represent the indirect effects of radiation, which result mainly from both 'OH and $e^{-}_{aq}$, while 10-eV electrons represent the direct effects of radiation. Electrons of 10 eV have larger $EF$s than gamma rays for DSB and interduplex CL in modified DNA. For the SSB formation, however, the $EF$ of the indirect effects of $\gamma$-rays is higher than LEEs. Nevertheless, there is no significant difference between these $EF$s for both electron- and $\gamma$-ray-irradiated samples (P-value $\geq$ 0.05).

Since the repair of a cluster damage is difficult and usually prone to error for a cell (Goodhead, 2006; Ward, J.F., Webb C.F., Limoli C.L., Milligan J.R., 1990; Ward, 1994), DSB and interduplex CL are believed to have considerable biological effects compared with other types of damage such as SSB (O'Driscoll and Jeggo, 2006; Obe, Johannes and Schulte-Frohlinde, 1992). In the presence of Pt-drugs, our results show that LEEs and $e^{-}_{aq}$ specifically induce DSB and interduplex CL in DNA. By increasing the
number of these electrons in the vicinity of Pt-DNA adducts, therefore, it is expected to enhance the formation of the cluster damages, leading to an increase in cell death and consequently to an improvement in the efficacy of Pt-based CCRT in clinic.

There are three strategies to produce more LEEs in radiotherapy: 1) high LET radiation, 2) molecules containing heavy metal atoms, and 3) Auger-electron emitting radionuclides (AE radionuclides). Since high LET radiations, including proton, alpha and heavy ion particles produce higher ionization in their tracks compared to low LET radiations (e.g., photon and electrons), they generate more LEEs. However, such radiations reduce the indirect effects of radiation due to the lower yields for the production of 'OH and $e_{aq}^-$ by radiations of high LET compared to those of low LET (Elliot, A.J., Bartels, D.M., 2009). G-value (i.e., produced molecules per 100 eV) for $e_{aq}^-$ generated by gamma radiations (LET $\leq$ 1 eV/nm), 1-MeV $^1$H (LET $\approx$ 10 eV/nm) and 5-MeV $^4$He (LET $\approx$ 100 eV/nm), for example, are 2.5, 0.48 and 0.26, respectively (LaVerne, 2004). Despite such considerable decrease in the number of $e_{aq}^-$, high LET radiation increases considerably the number of LEEs, which can not only compensate the lower quantity of $e_{aq}^-$, but also enhance substantially the formation of cluster damage. In the previous example, the yield for the generation of $e_{aq}^-$ becomes negligible after increasing LET by two orders of magnitude, whereas the number of LEEs increases by a factor of about 100 due to the similar increase in LET. Therefore, high LET radiations are expected to substantially increase the efficacy of Pt-based CCRT by enhancing the LEEs damages to modified DNA.

Radiotherapy with low LET radiations of a tumor tissue treated with heavy metal molecules (e.g., gold) also increases the number of LEEs, while maintaining the indirect effects of radiation, in contrast to high LET radiation, particularly the number of $e_{aq}^-$. Cross sections of Compton scattering and photoelectric effects for the interaction of therapeutic photon beams of 0.3 – 20 MeV with heavy metal molecules are one to two orders of magnitude larger than those of water. At these photon energies, the absorption cross section of gold, for example, is greater than water by factors of 10 – 40 (Sanche,
Irradiation of gold-cisplatin-DNA complex with 60 keV electrons has been reported to enhance substantially the formation of DSB (i.e., by a factor of 7.5) compared to cisplatin-DNA complex, while a very small enhancement in the SSB formation (i.e., by factors of 1.2 – 1.5) was observed (Zheng and Sanche, 2009). These results are in agreement with our study that LEEs markedly induce DSB in DNA modified by Pt-drugs.

Moreover, AE radionuclides emit a cascade of electrons (i.e., Auger electrons) with energies between near zero eV and a few keV via their decay by internal conversion and/or electron capture processes. Almost 50% of the Auger electrons are LEEs with a range of less than 2 nm; for instance, LEEs consist 53%, 51% and 40% of the Auger electrons emitted from the radionuclides of $^{111}$In, $^{77}$Br and $^{123}$I, respectively (Kassis, 2011). Theoretical studies have been reported that the absorbed dose of AE radionuclides is about 10 – 10000 KGY in a nanoscale volume (i.e., a radius of 2 nm) around the decay site (Kassis, Sastry and Adelstein, 1987; Michaud, Bazin and Sanche, 2013). The rest of the Auger electrons have a range of less than 500 nm, thus they deposit their energies inside the DNA and the molecules surrounding DNA, mainly including histone proteins and water. In the case of water, they enhance the production of both $^\cdot$OH and $e^-_{aq}$ in the immediate vicinity of DNA. Therefore, AE radionuclides can enhance the formation of cluster damage in DNA modified by Pt-drugs by both the direct and indirect effects of radiation, if they locate in the close proximity to Pt-DNA adducts.

III.2. Radiosensitization of modified DNA depends on the type of Pt-drugs

Results in Article 3 and 4 show that cisplatin, carboplatin and oxaliplatin enhance the formation of DNA damage by the direct effect of radiation, while their radiosensitization efficiencies are different. In the presence of carboplatin, the yields of DSB formation by 1.5-keV X-rays and electrons of 0.5, 10 and 10000 eV are higher than those in cisplatin-modified DNA by factors of 1.4, 1.4, 1.2, and 1.3, respectively. In the DNA modified by oxaliplatin, the corresponding factors respectively are 1.1, 1.7, 1.0, and 1.3 compared with cisplatin-DNA samples. Similar enhancement factors have also been observed for the formation of interduplex CL for DNA modified by either carboplatin or
oxaliplatin relative to those by cisplatin. At 10-eV electrons, for example, the presence of carboplatin or oxaliplatin in DNA results in the higher yields of interduplex CL compared to that for cisplatin-modified DNA, by factors of 1.4 and 1.9, respectively. These findings suggest that the relative efficiency of carboplatin and oxaliplatin in the sensitization of DNA towards radiation, particularly LEEs is higher than that of cisplatin. As explained in Article 3, each Pt-drug distorts DNA conformation in a distinctive manner, resulting in different chemical and physical properties, which modify the interaction of radiation.

In clinic, carboplatin, as a chemotherapeutic drug, is an appropriate alternative to cisplatin due to its lower side effects and its similar spectrum of activity (Kelland, 2007). Our results indicate that carboplatin, as a radiosensitizing agent, is superior to cisplatin for an equal number of Pt-adducts. Therefore, carboplatin in the combination with radiation is expected to have higher efficiency than cisplatin in therapeutic applications. Oxaliplatin has a safer toxicity profile than cisplatin and a different spectrum of activity relative to cisplatin and carboplatin. At the same concentration, oxaliplatin forms lower number of adducts since it has a lower reactivity than the two other Pt-drugs to DNA. In the chemotherapeutic implications, however, it has similar cytotoxicity to cisplatin (Woynarowski, et al. 2000). Our results indicate that oxaliplatin has higher radiosensitivity than cisplatin for an equal number of Pt-adducts; hence its combination with radiation is expected to have similar efficiency to cisplatin in therapeutic applications.

III.3. Radiosensitization of Pt-drugs depends on the number of Pt-DNA adducts

Our results in Article 3 indicate that radiosensitivity of DNA towards LEEs increases with the number of Pt-DNA adducts. The \textit{EFs} for the formation of DSB and interduplex CL by 10-eV electrons exhibit a biphasic increase with the quantity of Pt-DNA adducts, whereas the corresponding \textit{EFs} for the SSB formation show a monotonic rise with the adduct number. Such behaviors in the \textit{EFs} curves are similar between Pt-drugs, i.e., at small number of the adducts (i.e., less than \(3.1 \times 10^{-4}\) Pt-adduct per nucleotide), the rate of increase in the \textit{EFs} for DSB and interduplex CL is about one order of magnitude higher than those at larger quantity of the adducts. Fig. III.2 compares the \textit{EFs} for the formation of SSB, DSB and interduplex CL by \(\gamma\)-rays in an aqueous
solution of DNA and by 10-eV electrons in a solid film of DNA in the presence of cisplatin as a function of the number of Pt-DNA adducts. Similar to 10-eV electrons, the EF curves for DSB and interduplex CL by γ-rays exhibit a biphasic behavior with the quantity of Pt-DNA adduct. Table III.1 indicates the rate of increase in these EFs at two regions 1 and 2 (i.e., S₁ and S₂) corresponding to the number of Pt-DNA adducts less and higher than $3.1 \times 10^{-4}$ adducts, respectively. At the both regions, the rate of increase in the EFs for DSB and interduplex CL by 10-eV electrons are larger than those by γ-rays.

![Graphs showing EFs for DSB, interduplex CL, and SSB](image)

**Fig. III-2.** Enhancement factors in the yields of DSB (a), interduplex CL (b) and SSB (c) as a function of the number of Pt-DNA adducts per nucleotide for DNA modified by cisplatin and irradiated with 10-eV electrons and γ-rays. The fitted lines are based on a least-square regression analysis.
while the ratios \( R_{1,2} \) (i.e., the ratio between \( S_1 \) and \( S_2 \)) for \( \gamma \)-rays are larger than those for the electrons, mainly because of the small values for \( S_2 \) in the \( \gamma \)-irradiated samples compared to those in the LEE-irradiated films. These results suggest that the contribution of the indirect effect of ionizing radiation to the enhanced formation of radiation damage to modified DNA greatly decreases for the number of Pt-DNA adducts larger than \( 3.1 \times 10^{-4} \) adducts (i.e., at the region 2) compared with the direct effect of radiation. In contrast to 10-eV electrons, such a biphasic behavior in EFs curves has also been observed for the formation of SSB in the irradiated samples by \( \gamma \)-rays.

From this dependence of DNA radiosensitivity on the number of Pt-DNA adducts, it can be inferred that the mechanisms responsible for the radiation-induced damage to modified DNA are different at small and large number of Pt-DNA adducts. Previous studies on the platination of plasmid DNA has shown that at low concentration of cisplatin (i.e., less than \( 5 \times 10^{-4} \) cisplatin per nucleotide), interstrand CLs are the most probable cisplatin adducts in supercoiled configuration, while intrastrand CLs are the common adducts in the relaxed (i.e., nicked circular) and linear DNA forms (Bouayadi, Calsou, Pedrini and Salles, 1992; Vrana, Boudňy and Brabec, 1996). In the present study,

Table III.1. Enhancement Factors for the induction of SSB, DSB and interduplex CL by either 10-eV electrons or \( \gamma \)-rays as a function of the number of cisplatin-DNA adducts per nucleotide. \( S_1 \) and \( S_2 \) denote the slopes of the fitted lines to the Enhancement Factor curves at ratios less and more that \( 3.1 \times 10^{-4} \) Pt-adducts per nucleotide, respectively. \( R_{1,2} \) is the ratio of \( S_1 \) to \( S_2 \).

<table>
<thead>
<tr>
<th>DNA Damage*</th>
<th>Radiation</th>
<th>( S_1 \times 10^3 )</th>
<th>( S_2 \times 10^3 )</th>
<th>( R_{1,2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSB</td>
<td>10-eV Electrons</td>
<td>0.51 ± 0.03</td>
<td>0.51 ± 0.03</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>( \gamma )-rays</td>
<td>3.7 ± 0.3</td>
<td>0.18 ± 0.02</td>
<td>20.5 ± 2.8</td>
</tr>
<tr>
<td>DSB</td>
<td>10-eV Electrons</td>
<td>4.8 ± 0.2</td>
<td>0.54 ± 0.07</td>
<td>8.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>( \gamma )-rays</td>
<td>4.2 ± 0.5</td>
<td>0.29 ± 0.03</td>
<td>14.5 ± 2.3</td>
</tr>
<tr>
<td>CL</td>
<td>10-eV Electrons</td>
<td>3.84 ± 0.2</td>
<td>0.65 ± 0.06</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>( \gamma )-rays</td>
<td>1.85 ± 0.9</td>
<td>0.062 ± 0.01</td>
<td>29.8 ± 15.3</td>
</tr>
</tbody>
</table>
we found that the optimum radiosensitization of DNA, in terms of the damage per Pt-DNA adducts, lies below $3.1 \times 10^4$ Pt-adducts per nucleotide (i.e., 2 Pt-adducts per plasmid). This ratio agrees with those reported for the formation of interstrand CL between cisplatin and supercoiled plasmid DNA. Therefore, it appears that Pt-drugs interstrand CL makes DNA highly sensitive to both the direct and indirect effects of radiation.

To verify this hypothesis that is on the basis of the aforementioned studies for the preferential formation of Pt-drugs inter and intrastrand CLs (Bouayadi, Calsou, Pedrini and Salles, 1992; Vrana, Boudný and Brabec, 1996), we irradiated two different types of cisplatin-DNA samples containing either cisplatin interstrand or intrastrand CLs with γ-rays. For the DNA samples containing interstrand CL of cisplatin, complexes of supercoiled plasmid DNA with cisplatin were prepared at the average ratio of 2 Pt-adducts per plasmid. Owing to this small quantity of cisplatin, it is expected to form interstrand CL in the DNA. Such supercoiled DNA then converted to a circular form by the Nb.BtsI enzyme (BioLabs), which is a nicking endonuclease cleaving only one strand of a double-stranded DNA. For the DNA samples with intrastrand CL of cisplatin, a supercoiled DNA is first converted to a circular form by the enzyme, and then mixed with cisplatin solution to give a final ratio of 2 Pt-adducts. This circular DNA should have intrastrand CL of cisplatin, since cisplatin mainly forms intrastrand CL with a circular DNA (Vrana, Boudný and Brabec, 1996). Fig. III.3 indicates the lost of circular and the formation of linear DNA by $^{60}$Co γ-rays for the aqueous solution of Pt-DNA samples including either interstrand or intrastrand CLs. The yields of circular loss and linear formation (i.e., corresponding to DSB) for the irradiated samples with interstrand CL are greater by a factor of about 7.0 than those with intrastrand CL. These results, therefore, suggest that interstrand CL of Pt-drugs makes supercoiled DNA highly sensitive to radiation.

This finding that the type of Pt-DNA adducts influences the radiosensitivity of DNA could have applications in the design and development of new chemotherapeutic and radiosensitizing agents. Multinuclear platinum compounds, for example, are a type of Pt-drugs with positive charges and at least two platinum atoms, which mainly form
interstrand CL in DNA (Boulikas, Pantos, Bellis and Christofis, 2007). These Pt-drugs such as BBR3610 and BBR3571 are under investigation for clinical application. Since Pt-DNA adducts in the form of interstrand CL greatly enhance the radiosensitivity of DNA, the multinuclear Pt-drugs are expected to have higher efficiency than other Pt-drugs in CCRT.
IV. CONCLUSION

Sensitization of DNA towards both direct and indirect effects of radiation by Pt-drugs including cisplatin, carboplatin and oxaliplatin was investigated using a model of supercoiled plasmid DNA. In this research project, we determined the main secondary species, generated by primary high-energy ionizing radiation, responsible for enhanced damage to DNA modified by Pt-drugs, and elucidated the major molecular mechanisms underlying the radiosensitization. Furthermore, this project resulted in the development of a new method for the preparation of the complexes of plasmid DNA with Pt-drugs that are appropriate for the use in irradiation experiments, and a new molecular survival model to obtain absolute CSs for LEE damage to a macromolecule such as DNA within a nanometer-scale solid films.

The direct interaction of various radiations including 10000-, 10- and 0.5-eV electrons, and 1.5-keV X-rays with DNA modified by Pt-drugs indicates a preferential enhancement of DNA lesions, particularly the formation of cluster damages including DSB and interduplex CL. Comparison of the yields and EFs for the formation of SSB, DSB and interduplex CL by these radiations suggests that LEEs play a major role in the enhancement of DNA damage in the presence of Pt-adducts. The electrons of near-zero eV (i.e., 0.5 eV) are more efficient than the higher radiation energies in producing SSB in DNA, particularly in the presence of Pt-adducts, and are as efficient as 10-eV electrons and 1.5-keV X-rays in forming DSB in modified DNA. This finding is especially noteworthy in the view of the fact that 1.5-keV X-rays have LET much greater (i.e., by a factor of 400) than that for 0.5-eV electrons. Since 0.5 eV is well below that energy required for ionization and electronic excitation of any organic molecule, DEA via shape resonances is the major mechanism responsible for the enhanced damage in Pt-drug-modified DNA.

In a dilute aqueous solution of DNA, irradiation of cisplatin-modified DNA with $^{60}$Co $\gamma$-rays indicates an increase in the yields of SSB and DSB by factors of 1.9 and 2.2, respectively, relative to the irradiated unmodified DNA. When either $^{\cdot}$OH or $e_{aq}^{-}$ were scavenged, these enhancements decrease to factors between 1.2 - 1.6, and after
scavenging both 'OH and $e_a^-$, no enhancement in strand break formation was observed in modified DNA. These results suggest that the main species, created by the indirect effect of ionizing radiations, responsible for the enhanced damage in modified DNA are 'OH and $e_a^-$. Our data show that $e_a^-$ can induce both SSB and DSB in cisplatin-modified DNA, but not in unmodified DNA. In the presence of Pt-adducts, $e_a^-$ mainly contributes to the induction of DSB (i.e., 86% of the enhanced formation of DSB), while 'OH mostly increase the production of SSB (i.e., 57% of the enhanced formation of SSB).

Radiosensitization of modified DNA to both direct and indirect effects of ionizing radiation increases with the number of Pt-adducts. Optimum radiosensitization, in terms of damage per Pt-adduct lies below ratios of $3.1 \times 10^4$ Pt-adducts per nucleotide. Moreover, the relative efficiency of carboplatin and oxaliplatin in DNA sensitization towards the electrons and X-rays is higher than cisplatin, particularly for the formation of DSB and interduplex CL. With respect to the similarities in the reactive forms of the Pt-drugs, the type of Pt-adducts is suggested to be responsible for the observed different efficiencies in DNA radiosensitization by Pt-drugs. Our data demonstrate that the radiosensitization effect of Pt-drug interstrand CL is substantially higher than those of intrastrand CL.
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