*Anal. Chem.* **2002,** *74,* 6163−6169

# DNA Damage Detection Technique Applying Time-Resolved Fluorescence Measurements

Gonzalo Cosa,[†] Anne L. Vinette,[†] J. R. N. McLean,[‡] and J. C. Scaiano*,[†]

*Department of Chemistry, University of Ottawa, 10 Marie Curie, Ottawa, Ontario, K1N 6N5, Canada, and Radiation Protection Bureau, Health Canada, 775 Brookfield Road, Ottawa, Ontario, K1A 1C1, Canada*

**A novel DNA damage detection technique based on the characteristic fluorescence lifetimes exhibited by PicoGreen−single-stranded DNA and −double-stranded DNA complexes is employed to establish the damage produced on DNA isolated from sheep white blood cells following $\gamma$ radiation. This technique, which incorporates key concepts such as alkaline unwinding buffers and higher unwinding rates of damaged DNA compared to undamaged DNA, allows for the differentiation of DNA damage resulting from doses of $\gamma$ radiation in the 0−100-Gy range, with the potential of analyzing samples consisting of as little as $10^4$ cells. Experiments were carried out using commercial DNA sources as well as DNA isolated from sheep white blood cells, suggesting its potential for use with isolated DNA from virtually any eukaryotic cell.**

Damage to DNA in its biological environment has been associated with alterations in DNA sequence, aberrant gene expression, increased mutation rates, cell transformation, and development of cancer. DNA damage can arise as a result of inherent DNA chemical instability, as a byproduct of endogenous cellular processes such as oxidative metabolism,[1] or by exposure to exogenous genotoxic agents.[2] The disruption of the structural or functional integrity of DNA is therefore an important first step in establishing the carcinogenic potential of a chemical or physical agent.

Chemically induced damage to DNA will render a weakened polymer structure at the site of the attack (alkali labile site (ALS)), and upon appropriate chemical treatment, these damaged sites will give rise to strand breaks (SB),[3,4] eventually causing DNA unwinding.[5] Many analytical techniques currently employed for quantifying DNA damage rely on the conversion of double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) following an unwinding protocol. These techniques, among many others, include the comet assay,[6−9] the fluorometric analysis of DNA unwinding,[10,11] the alkaline filter elution,[12] and the temperature unwinding technique.[13]

Previous work from our group indicates that the amount of ssDNA and dsDNA present in solution can be measured by the fluorescence lifetime of dye−DNA complexes.[14] We have previously found that PicoGreen is the best dye (out of nine commercial DNA dyes explored) suited for this purpose.[15] PicoGreen belongs to a family of recently patented[16,17] fluorescent dyes, derived from unsymmetric cyanine dyes, which exhibit a high increase (∼1000-fold) in their fluorescence quantum yields upon binding to dsDNA compared to free in solution, making them very sensitive probes for DNA detection.[18,19] Following excitation of the trans form to the excited trans-singlet state, rotation along the methine bridge converts PicoGreen into the excited perpendicular singlet state, which decays nonradiatively through internal conversion, to the perpendicular ground state.[20,21] Upon intercalation into DNA base pairs, this rotation along the methine bridge is restricted and PicoGreen is forced to dissipate its energy predominantly through emission via fluorescence, which explains its high sensitivity as a dsDNA sensor.

Other techniques developed to assess DNA damage employ fluorescence as the main tool for the quantification of ssDNA. Many of these techniques use PicoGreen as the fluorophore; however, steady-state rather than time-resolved measurements have been employed in all these cases.[13,22] The fluorescence lifetime is an intensive property; i.e., it is not dependent on the

* Corresponding author. E-mail: tito@photo.chem.uottawa.ca.
[†] University of Ottawa.
[‡] Health Canada.

(1) Loeb, L. *Cancer Res.* **1989**, *49*, 5489−5496.
(2) Mullaart, E.; Lohman, P. H. M.; Berends, F.; Vijg, J. *Mutat. Res.* **1990**, *237*, 189−210.
(3) Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109−1151.
(4) Pogozelski, W. K.; Tullius, T. D. *Chem. Rev.* **1998**, *98*, 1089−1107.
(5) Frenkel, k.; Klein, C. B. *J. Chromatogr.* **1993**, *618*, 289−314.
(6) Olive, P. L. *Int. J. Radiat. Biol.* **1999**, *75*, 395−405.
(7) Horváthová, E.; Slamenová, D.; Hlincíková, L.; Mandal, K. T.; Gábelová, A.; Collins, A. R. *Mutat. Res.* **1998**, *409*, 163−171.
(8) Rojas, E.; Lopez, M. C.; Valverde, M. *J. Chromatogr., B* **1999**, *722*, 225−254.
(9) Collins, A. R.; Dobson, V. L.; Dusinská, M.; Kennedy, G.; Stetina, R. *Mutat. Res.* **1997**, *375*, 183−193.
(10) Birnboim, H. C.; Jevcak, J. J. *Cancer Res.* **1981**, *41*, 1889−1892.
(11) Baumstark-Khan, C.; Hentschel, U.; Nikandrova, Y.; Krug, J.; Horneck, G. *Photochem. Photobiol.* **2000**, *72*, 477−484.
(12) Kohn, K. W. *Pharmacol. Ther.* **1991**, *49*, 55−77.
(13) Rogers, K. R.; Apostol, A.; Madsen, S. J.; Spencer, C. W. *Anal. Chem.* **1999**, *71*, 4423−4426.
(14) Cosa, G.; Focsaneanu, K.-S.; McLean, J. R. N.; Scaiano, J. C. *Chem. Commun.* **2000**, 689−690.
(15) Cosa, G.; Focsaneanu, K.-S.; McLean, J. R. N.; McNamee, J. P.; Scaiano, J. C. *Photochem. Photobiol.* **2001**, *73*, 585−599.
(16) Haugland, R. P.; Yue, S. T.; Millard, P. J.; Roth, B. L. US5436134, 1995.
(17) Yue, S. T.; Singer, V. L.; Roth, B. L.; Mozer, T. J.; Millard, P. J.; Jones, L. J.; Xiaokui, J.; Haugland, R. P.; World Intellectual Property Organization W096/13552, 1996.
(18) Netzel, T. L.; Nafisi, K.; Zhao, M.; Lenhard, J. R.; Johnson, I. *J. Phys. Chem.* **1995**, *99*, 17936−17947.
(19) Carlsson, C.; Larsson, A.; Jonsson, M.; Albinsson, B.; Nordén, B. *J. Phys. Chem.* **1994**, *98*, 10313−10321.
(20) Saltiel, J. *J. Am. Chem. Soc.* **1967**, *89*, 1036−1037.
(21) Gilbert, A.; Baggott, J. *Essentials of Molecular Photochemistry*; Blackwell Scientific Publications: Oxford, 1991.

10.1021/ac025888j CCC: $22.00 
Published on Web 11/14/2002

amount of fluorophore and it is unique for a dye within a given environment. Lifetime measurements thus render reliable results without the need for blanks. The latter is not true for steady-state fluorescence experiments, where it is necessary to compare the intensities from stained damaged DNA samples to stained control samples.

In what follows, we describe a new technique, based on fluorescence lifetimes, developed to measure DNA damage. We also address the potential effect the viscosity of the DNA support materials may have on fluorescence lifetimes of the dye−DNA complexes. We evaluate the sensitivity of our technique by analyzing results obtained using different unwinding protocols on undamaged calf thymus DNA (CT-DNA). Finally, we discuss the results obtained using this novel technique with DNA isolated from sheep white blood cells (WBC) following different doses of $\gamma$ radiation.

## EXPERIMENTAL SECTION

**Materials.** Sheep blood was obtained from the Animal Diseases Research Institute at Agriculture Canada (Ottawa, ON, Canada). PicoGreen nucleic acid stain was obtained from Molecular Probes (Eugene, OR) and kept at −20 °C until used. Hank's balanced salt solution without phenol red, 0.1 $\mu$m filtered (HBSS), was from Gibco BRL Life Technologies (Grand Island, NY). CT-DNA (type I), salmon testes DNA (type III) (ST-DNA), Trizma preset crystals (reagent grade, pH 7.4), disodium ethylenediamine tetraacetate ($Na_2EDTA$), phosphate-buffered sodium heparin at 500 units/mL, fluorescein, fluorescein diacetate, ethidium bromide (EtBr), and *N*-lauroylsarcosine sodium salt (SLS) were obtained from Sigma Chemical Co. (St. Louis, MO). Agarose high-strength, analytical grade, ultrapure DNA grade (low-melting point agarose) was obtained from Bio-Rad Laboratories (Hercules, CA). Dimethyl sulfoxide (DMSO), tris(hydroxymethylamino)methane (Tris base), acetone, sodium tetraethylenediamine tetraacetate (EDTA), methanol, sodium phosphate monobasic ($NaH_2PO_4 \cdot 2H_2O$), and sodium phosphate dibasic ($Na_2HPO_4$) were obtained from Fischer Scientific (Fairlawn, NJ). Isooctylphenoxypolyethoxyethanol (Triton X-100), sodium chloride, and potassium hydroxide were from BDH (Toronto, ON, Canada). The TAE buffer, (0.4 M Tris acetate, 0.2 M glacial acetic acid, 0.1 M EDTA), 10×, pH 8.4, was from Roche Diagnostics (Manheim, Germany). Wright-Giemsa stain was obtained from EM Science (Gibbstown, NJ). All solvents and chemicals were used without further purification.

**Solution Preparation.** All solutions were prepared with 18-MΩ water ($ddH_2O$) (Milli-Q plus PF unit, Millipore Corp., Bedford, MA). $ddH_2O$ for red blood cell lysis was sterilized by passing it through a 0.2-$\mu$m filter (Acrodisc, low protein binding, Gelman Sciences, Ann Arbor, MI). The 2× saline rescue solution, also sterilized in the same way as the water for red blood cell lysis, consisted of 1.8% NaCl in $ddH_2O$. Fluorescein diacetate was prepared at 5 mg/mL in acetone. The EtBr solution was prepared at 200 $\mu$g/mL in HBSS. The working viability stain was prepared by adding 50 $\mu$L of the EtBr solution and 7.5 $\mu$L of the fluorescein diacetate solution to 1.2 mL of HBSS. The PBS solution consisted of 58 mM $Na_2HPO_4$, 17 mM $NaH_2PO_4 \cdot 2H_2O$, and 68 mM NaCl in $ddH_2O$ to achieve a pH of 7.4. The agarose solution was prepared in PBS pH 7.4 to 0.75% consistency (w/v). The lysis buffer consisted of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% SLS in $ddH_2O$, adjusted to pH 10; 1% Triton X-100 and 10% DMSO were added to the lysis buffer immediately before use, to assist in the removal of cellular proteins. The Tris buffer consisted of 10 mM Trizma preset crystals, 1 mM $Na_2EDTA$, and 100 mM NaCl in $ddH_2O$ to achieve a pH of 7.4. Various concentrations of the unwinding buffer were prepared in $ddH_2O$, ranging from 0.005 to 0.1 M KOH. The Giemsa stain was prepared by adding 5 mL of the Giemsa solution to 100 mL of $ddH_2O$. The 1× TAE buffer was prepared by making a 1:10 dilution of the 10× TAE stock solution. The PicoGreen staining solution was prepared by adding 196 $\mu$L of PicoGreen to 10 mL of Tris buffer solution (∼1:50 dilution of the stock solution supplied by Molecular Probes to obtain a 5.9 $\mu$M solution), and the solution was stored at 4 °C and protected from light.

**Experiments with Commercial DNA on Solid Supports.** CT-DNA solutions were prepared in a Tris buffer at pH 7. The ssDNA was obtained after boiling a dsDNA solution for 30 min followed by immediate immersion on an ice bath. Complete denaturation was confirmed by comparing the absorption ratio at 260 nm for the heated (ssDNA) and nonheated (dsDNA) samples. A value of 1.32 was obtained, in good agreement with the literature value.[23] The DNA solution (20 $\mu$L, 1 mM) was mixed with 20 $\mu$L of the PicoGreen staining solution (100 $\mu$M) and then added to 360 $\mu$L of 0.75% agarose.

**Isolation of White Blood Cells from Sheep Whole Blood.** All plasticware used during the isolation process was made of polypropylene, and high-binding plastics, such as polystyrene, were avoided as they bind cells to the centrifuge tube walls. A volume of 100 mL of sheep whole blood (WB) was collected in a culture flask containing 10 mL of phosphate-buffered sodium heparin. The sheep WB was dispensed into centrifuge tubes (15 mL of WB × 8 centrifuge tubes) and kept on ice for 30 min. Red blood cells were lysed with 15 mL of ice-cold $ddH_2O$ for no longer than 30 s, and then 15 mL of ice cold 2× saline solution was added to stop the red blood cell lysis and restore the cellular osmotic pressure. The mixtures were centrifuged for 1 h at 1500 rpm. The supernatants were decanted, and the red blood lysis was repeated (5 mL of $ddH_2O$ and 5 mL of 2× saline solution) to remove the remaining red blood cells. The WBC were collected by centrifugation for 15 min at 1500 rpm. The supernatants were decanted, and the WBC pellets were resuspended in 2.5 mL of HBSS and pooled.

**Cell Viability and Concentration Determination.** To determine the cell concentration and viability, an aliquot of the cell suspension (50 $\mu$L) was taken, mixed with viability stain (50 $\mu$L), dispensed onto a hemacytometer, and examined with a fluorescence microscope (Olympus BX-60, Olympus Optical Co.) at 20× (UPlanAPO objective) fitted with a U-MNB (blue band) filter cube. Under these conditions, nonviable cells appeared red while viable cells fluoresced green due to the conversion of fluorescein diacetate to fluorescein by membrane esterases in metabolically competent (viable) cells.[24] This method allowed for the concentra-

(22) Elmendorff-Dreikorn, K.; Chauvin, C.; Slor, H.; Kutzner, J.; Batel, R.; Muller, W. E.; Schroder, H. C. *Cell. Mol. Biol.* **1999**, *45*, 211−218.

(23) Gallagher, S. R. In *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds.; Greene Pub. Associates and Wiley-Interscience: New York, 1994; Vol. 3, pp A.3D.1−A.3D.8.

(24) Strauss, G. H. *Mutat. Res.* **1991**, *252*, 1−15.

tion determination in cells per milliliter.

To determine whether the cells had been harmed during isolation manipulations, a Giemsa stain test was performed. A drop of the cell suspension was dispensed onto a 75 mm × 25 mm microscope slide and allowed to dry. The slide was then submerged into the Giemsa staining solution for 5−10 min, removed, and allowed to dry. Once dried, the slide was submerged into methanol for 5−10 min, removed, and allowed to dry. The dried slide was examined by bright-field microscopy (Nikon Labophot, Nikon Canada Inc., Mississauga, ON, Canada), at 60× (PlanApo objective).

**DNA Concentration Determination.** To verify the actual concentration of the WBC suspension isolated from sheep WB, a DNA standard curve was constructed against which a sample of the cell suspension was analyzed. The DNA standard curve was constructed following a protocol detailed by Molecular Probes using their PicoGreen dsDNA quantitation reagents and kits. All solutions were prepared using polypropylene plasticware.

**Irradiation.** The WBC suspension was centrifuged for 30 min at 1500 rpm. The supernatant was discarded, and the pellet was resuspended in HBSS to obtain a cell concentration of $60 \times 10^6$ cells/mL. The WBC suspension was divided, dispensed into glass vials (as many vials as there are $\gamma$ radiation doses), and put on ice. For $\gamma$ radiation doses of 1−10 Gy, irradiations were carried out using the Gammacell40 reactor (Atomic Energy of Canada Ltd., Radiochemical Co.) $^{137}$Cesium source with a dose rate of 0.955 Gy/min. For $\gamma$ radiation doses of 25, 50, and 100 Gy, irradiations were carried out using the Gammacell220 reactor (Atomic Energy of Canada Ltd., Radiochemical Co.) $^{60}$Cobalt source with a dose rate of 38.462 Gy/min. All irradiations were performed with the glass vials, containing the cell suspension, on ice.

**Casting.** Gelbond film (agarose gel support medium from BioWhittaker Molecular Applications, Rockland, ME) is a flexible support consisting of an agarose-coated polyester sheet. Films were cut in 6 cm × 10 cm dimension. For each dose of $\gamma$ radiation, a volume of 50 $\mu$L of WBC suspension was mixed with 125 $\mu$L of 0.75% agarose. An aliquot of 150 $\mu$L of the cell suspension (containing at least 1 million cells) was dispensed as a bead onto a Gelbond film (6 beads/film i.e., three radiation doses in duplicate).

**Lysing and Unwinding.** Once the gels had solidified, they were submerged into a 150-mm Petri dish containing 50 mL of ice-cold lysis buffer and kept at 4 °C overnight. After at least 14 h,[25] the lysis buffer was removed and the gels were rinsed with ice cold HBSS. The gels were then submerged into a 150-mm Petri dish containing 50 mL of ice cold unwinding buffer and kept at 4 °C for 0−45 min (depending on the experiment). Once the unwinding time had lapsed, the gels were rinsed with ice-cold HBSS, then 50 mL of fresh ice-cold HBSS was dispensed onto the gels, and they were kept at 4 °C until staining.

**Staining.** Each gel was carefully peeled off of the Gelbond film with a spatula and placed into a well of a 24-well culture plate. The gels were stained by adding 150 $\mu$L of the PicoGreen staining solution and kept at 4 °C overnight ($\sim 8.9 \times 10^{-10}$ mol of PicoGreen was added to each gel). The gels contained 6 $\mu$g of DNA (equivalent to 9.5 nmol of base pairs) and were exposed to ~0.89 nmol of PicoGreen resulting in a dye:DNA ratio = 1:10. Previous results show that high dye/DNA ratios (i.e., dye:DNA > 1:10) are not operational due to the possibility of intermolecular interactions between two dye molecules, which affect the kinetic laws employed in the data analysis. It is therefore important to work under conditions of low dye/DNA ratios.[14]

**Time-Resolved Measurements.** Each gel was carefully placed into the tip of a glass NMR tube, ready to be measured. Fluorescence time-resolved measurements for short-lived singlet states were carried out using the third harmonic (355 nm) pulse from a Continuum PY-61 Nd:YAG laser (35 ps, ≤4 mJ/pulse) as the excitation source. A Hamamatsu C4334 streak camera was used for time-resolved fluorescence detection and data acquisition.[26]

**Time-Resolved Data Analysis.** The results obtained following 355-nm irradiation of a sample show a virtually pure monoexponential decay with a lifetime of 4.5 ns for the PicoGreen−dsDNA complex. The decay obtained for the PicoGreen−ssDNA complex is biexponential, with lifetimes of 1.16 (51%) and 3.09 ns (49%) with the weight of each exponential given in the parentheses.[14]

Provided that PicoGreen−ssDNA and PicoGreen−dsDNA complexes behave independently of each other, we have shown that a triexponential decay best fits this system (eq 1).[14] In eq 1,

$$I = a_{ds} e^{-0.22t} + \frac{(1 - a_{ds})}{2} (e^{-0.86t} + e^{-0.32t}) \qquad (1)$$

$I$ is the fluorescence intensity, $a_{ds}$ is the preexponential factor for dsDNA (i.e., its nominal abundance), 0.22 is the reciprocal of its lifetime ($ns^{-1}$), 0.86 and 0.32 are the corresponding parameters for ssDNA, and $t$ is the time.[14]

The percentage of dsDNA can be obtained from $a_{ds}$ corresponding to the dsDNA rate constant according to eq 2.[14] As the

$$\% \text{ dsDNA} = 100\left(\frac{a_{ds} - 0.048}{0.80}\right) \qquad (2)$$

background noise contribution is specific to the instrument used to measure the fluorescence lifetimes, slight deviations from these fits may occur with different instruments.[14]

The steps previously described are summarized in Figure 1.

## RESULTS

**Spectroscopy of Support Materials.** The protocols currently employed for the manipulation of cells, with the aim of isolating the DNA from cellular components, require the use of support materials. Many reports describe the treatment of cells, embedded in agarose, with a lysis buffer to remove the cellular membrane, proteins and RNA, leaving the DNA backbone intact.[5−9,22,27] The isolated DNA is then subjected to an unwinding buffer in order to convert the DNA damage into a measurable property (i.e., single-strand breaks (SSBs)). In our search toward the development of a new DNA damage detection technique based on dye−DNA complex fluorescence lifetimes, it was important to establish

(25) Banáth, J. P.; Kim, A.; Olive, P. L. *Radiat. Res.* **2001**, *55*, 564−571.

(26) Mohtat, N.; Cozens, F. L.; Scaiano, J. C. *J. Phys. Chem. B* **1998**, *102*, 7557−7562.

(27) Reinhardt-Poulin, P.; McLean, J. R.; Deslauriers, Y.; Gorman, W.; Cabat, S.; Rouabhia, M. *Photochem. Photobiol.* **2000**, *71*, 422−425.

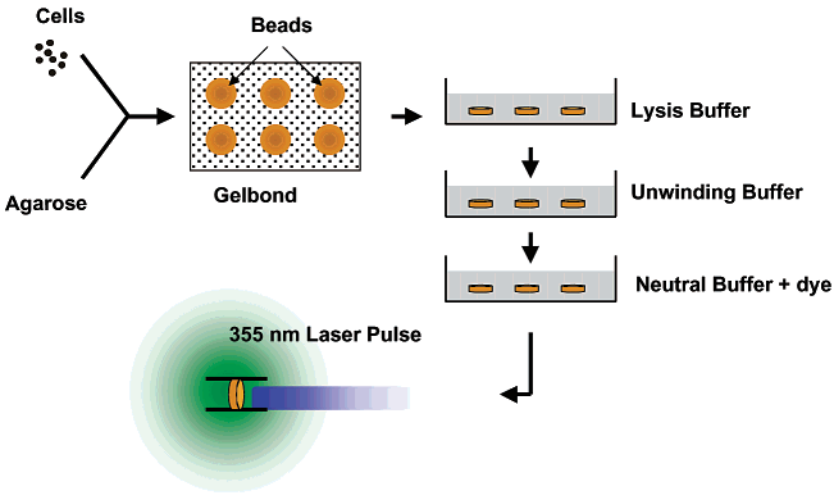


Figure 1. Steps of the protocol employed for time-resolved fluorescence measurements using DNA isolated from living cells following damage from $\gamma$ radiation.

the effect of agarose on the PicoGreen−DNA complex fluorescence decay, since our technique also required the use of this material in which to embed the cells. It was also necessary to establish to what extent the background signals, from either the fluorescence of the solid matrix employed in supporting the agarose gels (i.e., Gelbond film) or the gels themselves, interfered with the kinetic traces for dye−DNA complexes.

The solvent viscosity dependence of the lifetime for cyanine−stilbene dyes has been reported and is a concern when a DNA damage detection technique is being established since, during the analysis, the DNA will be fixed in a viscous support material (i.e., agarose).[18,28,29] We measured the lifetime of PicoGreen−ssDNA and PicoGreen−dsDNA complexes embedded in an agarose gel. We note that, in both cases, the fluorescence decay traces obtained with PicoGreen−DNA complexes embedded in agarose match those obtained with PicoGreen−DNA complexes in PBS (data not shown).

Our protocol involves mixing the cell suspension with agarose and casting onto a solid matrix, commercially known as Gelbond film. The gels remain fixed to this support unless they are physically removed. To establish the compatibility of this material with our measurements, we verified its absorbance as well as its steady-state and time-resolved fluorescence characteristics.

The absorption of Gelbond film tails on the visible part of the electromagnetic spectrum, presenting a considerable absorption at the excitation wavelength employed, i.e., 355 nm. Furthermore, its fluorescence emission, with a maximum at ~410 nm and extending out to 600 nm, presents a lifetime value that interferes with the signal from the PicoGreen−DNA complexes. Thus, this support material cannot be used in the later stages of a DNA damage detection technique, i.e., when time-resolved fluorescence measurements are necessary.

**Effects of $\gamma$ Radiation and Unwinding Time on DNA.** To analyze DNA damage, the proper conditions in terms of cell lysis and DNA unwinding must initially be established. While it is important to find conditions for unwinding the damaged DNA, at the same time it is desirable that little or no unwinding occurs in an undamaged sample.

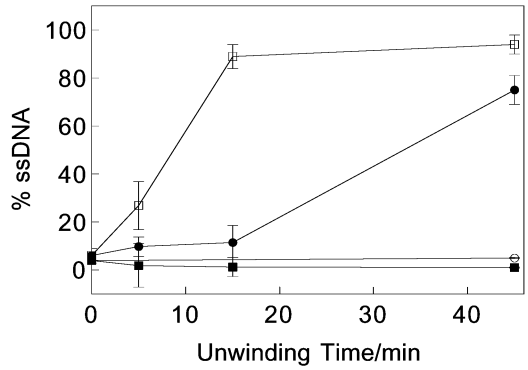


Figure 2. Percentage of ssDNA obtained with CT-DNA following no $\gamma$ radiation with different unwinding times and unwinding buffers of (□) 0.1, (●) 0.05, (■) 0.01, and (○) 0.005 M. The samples consisted of a 100 $\mu$L of agarose gel containing 9 nmol of CT-DNA base pairs.

We have employed hydroxyl radicals ($HO^{\bullet}$), generated following $\gamma$ irradiation of water, as the source of DNA damage. The effects of $\gamma$ radiation in aqueous solutions containing cellular material are well established.[30,31] Following a dose of 1 Gy (equivalent to 1 J of energy deposited in 1 kg of substance), 1000 SSBs and 40 double-strand breaks (DSBs) are produced in the DNA of one cell.[30,31]

Once the method for producing DNA damage had been established, appropriate conditions for DNA unwinding needed to be determined; therefore, we conducted the initial experiments with commercially available sources of the biopolymer. The results presented in Figure 2 show the relative amount of ssDNA produced in CT-DNA following various unwinding times and different unwinding conditions; this was determined from the decay traces analyzed according to eqs 1 and 2 (data not shown). We observe that at low concentration of unwinding buffer (aqueous KOH), such as 0.005 and 0.01 M, no unwinding, i.e., no

(28) Sauerwein, B.; Murphy, S.; Schuster, G. B. *J. Am. Chem. Soc.* **1992**, *114*, 7920−7922.
(29) Sczepan, M.; Rettig, W.; Bricks, Y.; Slominski, Y. L.; Tolmachev, A. I. *J. Photochem. Photobiol. A: Chem.* **1999**, *124*, 75−84.
(30) Ward, J. F. In *Progress in Nucleic Acid Research and Molecular Biology*; Academic Press: New York, 1988; Vol. 35, pp 95−125.
(31) Goodhead, D. T. *Int. J. Radiat. Biol.* **1994**, *65*, 7−17.

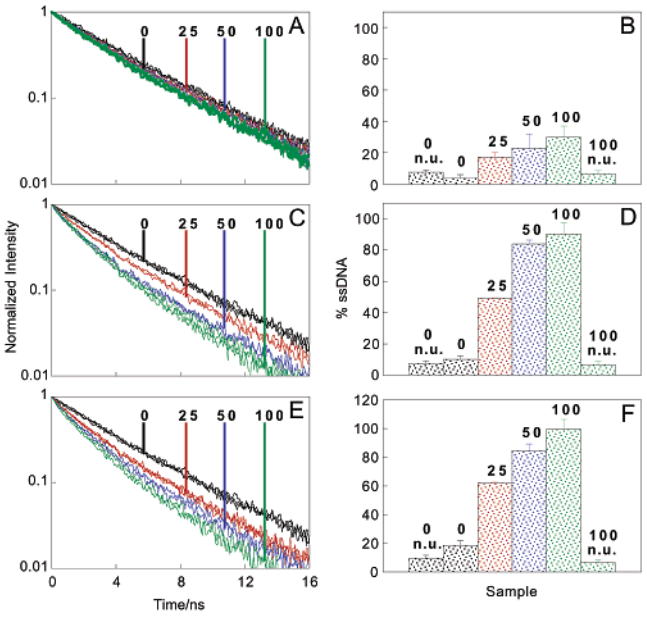


Figure 3. (A, C, E) Fluorescence decay profiles obtained for PicoGreen−DNA complexes using DNA isolated from sheep WBC. (B, D, F) Percentage of ssDNA produced, after unwinding, calculated from eqs 1 and 2 (obtained from the respective fluorescence decay traces in (A), (C), and (E)). Unwinding conditions: (A) and (B) 0.01 M KOH and 2-min unwinding; (C) and (D) 0.05 M KOH and 5-min unwinding; (E) and (F) 0.05 M KOH and 10-min unwinding.

ssDNA, is produced within experimental error. The amount of ssDNA becomes noticeable only after unwinding for 45 min with 0.05 M KOH buffer. A 2-fold increase in the concentration of unwinding buffer (0.1 M) dramatically changes our results, where complete unwinding of DNA is observed after 15 min.

**DNA Damage Detection in White Blood Cells.** The results obtained with CT-DNA (vide supra) allowed us to establish a range of unwinding conditions to be applied on DNA isolated from WBC. WBC extracted from sheep WB were irradiated with $\gamma$ radiation with doses ranging from 0 to 100 Gy. The samples were then subjected to three different unwinding conditions: (1) unwinding with a 0.01 M KOH buffer for 20 min, (2) unwinding with a 0.05 M KOH buffer for 5 min, and (3) unwinding with a 0.05 M KOH buffer for 10 min. The decay traces obtained for the PicoGreen−DNA complexes, and the amount of ssDNA quantified for each condition according to eqs 1 and 2, are presented in Figure 3.

The values of ssDNA, extracted from eqs 1 and 2, are also presented in Table 1. The trend observed in Figure 3A and B was expected; however, the results are within experimental error (defined by the error bars on the graphs). When the unwinding buffer concentration is increased to 0.05 M (Figure 3C−F) we observe an increased sensitivity, particularly for the detection of low levels of DNA damage. We were able to measure DNA damage produced with a $\gamma$ radiation dose as low as 5 Gy with these conditions (see Figure 4 and Table 1).

## DISCUSSION

Previous results showed the possibility of employing fluorescence lifetimes of PicoGreen−DNA complexes to determine ssDNA to dsDNA ratios.[14,15] In this work, we pursue the objective of establishing a technique for the measurement of DNA damage with that approach. It is therefore necessary to establish the proper

Table 1. Percentage of ssDNA Measured in DNA Isolated from WBC Following Various $\gamma$ Radiation Doses and Unwinding Times[a]

| unwinding conditions | | % ssDNA | | | | | |
|---|---|---|---|---|---|---|---|
| [buffer]/M | time/min | 0 Gy | 5 Gy | 10 Gy | 25 Gy | 50 Gy | 100 Gy |
| 0 | 0 | 7.4 | | | | | 6.7 |
| 0.01 | 20 | 3 | | | 17 | 23 | 30 |
| 0.05 | 5 | 10 | | | 49 | 84 | 90 |
| 0.05 | 10 | 18 | 32[b] | 44[b] | 62 | 85 | 100 |

[a] The data were extracted, according to eqs 1 and 2, from the results presented in Figures 3 and 4. Each value is the mean of three independent measurements. [b] These values are the mean of six independent measurements.

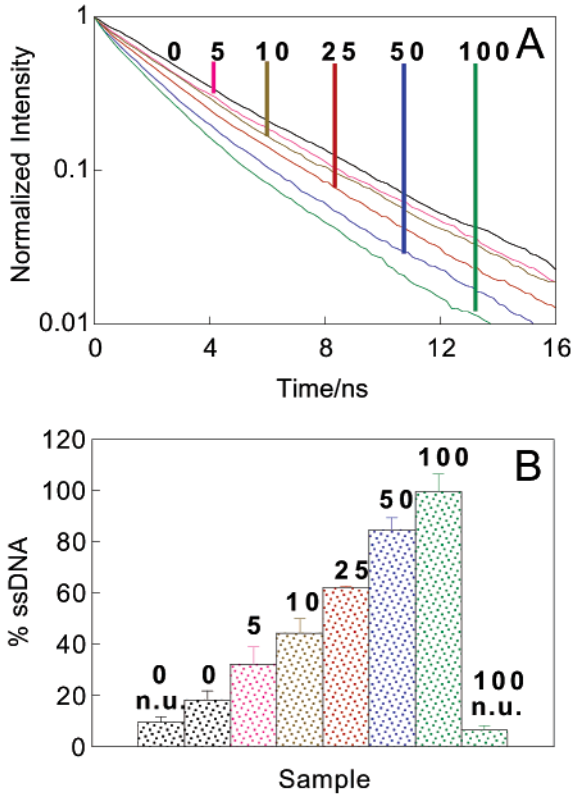


Figure 4. (A) Fluorescence decay profiles of PicoGreen−DNA complexes using DNA isolated from sheep WBC following 0, 5, 10, 25, 50, and 100 Gy of $\gamma$ radiation and unwound for 10 min with a 0.05 M unwinding buffer. Only one trace (smoothed) is presented for each condition. (B) Percentage of ssDNA produced, after unwinding, calculated from eqs 1 and 2 (obtained from the respective fluorescence decay traces in (A)).

conditions and potential of the method. In this sense, we have explored the minimum amount of analyte necessary for a measurement, as well as the minimum damage detectable in terms of SB, to establish a field of application for the technique.

As the source of DNA damage, we have chosen different doses of $\gamma$ radiation. This damage is mostly mediated by $HO^{\bullet}$ produced following water radiolysis.[3,4,32,33] A recent publication reports the

(32) Dizdaroglu, M.; von Sonntag, C.; Schulte-Frohlinde, D. *J. Am. Chem. Soc.* **1975**, *97*, 2277−2278.
(33) Doddridge, Z. A.; Cullis, P. M.; Jones, G. D. D.; Malone, M. E. *J. Am. Chem. Soc.* **1998**, *120*, 10998−10999.

quantification of SSBs and DSBs produced in DNA, as well as the number of base modifications resulting in ALS following administration of a given dose of this type of radiation.[34]

**Sensitivity to DNA Damage.** The sensitivity of this technique to DNA damage is established by two factors; the first is the minimum measurable difference in ssDNA to dsDNA ratio, which is intrinsic to the instrument response and the photophysics of the dye−DNA system. The second factor is dependent on the biggest differentiation in the ratio of the two forms of DNA produced, for a given dose of $\gamma$ radiation, and it is a function of the protocol employed for the unwinding of the DNA isolated from the WBC.

We optimized the unwinding conditions using commercially available forms of DNA (CT-DNA), and we established, from the results presented in Figure 2, that a 0.05 M KOH buffer provided the necessary sensitivity for this technique. In fact, a 5-fold decrease in the unwinding buffer concentration yielded no unwinding, whereas a 2-fold increase yielded complete DNA unwinding after 15 min for an undamaged sample.

We tested these conditions with DNA isolated from irradiated and nonirradiated sheep WBC. From the analysis of the data, presented in Figures 3 and 4 and Table 1, the optimized conditions for the detection of DNA damage from 0 to 100 Gy of $\gamma$ radiation is given by 10 min of unwinding with a 0.05 M KOH buffer. It is clear that an unwinding buffer concentration of 0.01 M is not strong enough since little differentiation between the $\gamma$ radiation doses is discernible even after 20 min of unwinding (Figure 3A and B). With an unwinding buffer concentration of 0.05 M and 5-min unwinding, we observe that the unwinding is not complete and that these conditions are not optimal since the maximum detection range of the technique (given by a value of 100% ssDNA) has not been reached (Figure 3C and D).

The observed trend using milder conditions of the unwinding buffer indicates that they can be successfully employed when analysis of stronger damage is needed. This is evidenced in Figure 5, which is a compilation of the data from Figure 3. We can also infer from this plot that whenever lower amounts of DNA damage need to be analyzed, stronger unwinding conditions should be employed.

**Relevance of the Doses Measured.** It is important to establish the range of DNA damage resulting from $\gamma$ radiation that can successfully be measured. Our present results show that this technique is capable of differentiating DNA damage as low as that introduced by a dose of 5 Gy of $\gamma$ radiation. Under these conditions, up to 5000 SSBs and 200 DSBs are introduced in one cell.[30,31] This dose of $\gamma$ radiation is relatively high (for humans); however, it has been reported that, during the Chernobyl accident, some people received doses as high as 10 Gy of radiation and have survived for more than 14 years.[35] We believe that, at the present time, our technique can be employed as a fast diagnostic tool for the detection of DNA damage following exposure to high doses of nuclear radiation.

Radiotherapy treatments usually involve doses of 2 Gy.[22] These doses could be detected by our technique, by selecting stronger unwinding conditions that would result in complete DNA unwinding (i.e., 100% ssDNA). Using more rigorous unwinding conditions, it could be possible to accurately differentiate DNA damage following doses of $\gamma$ radiation from 0 to 5 Gy with this technique.

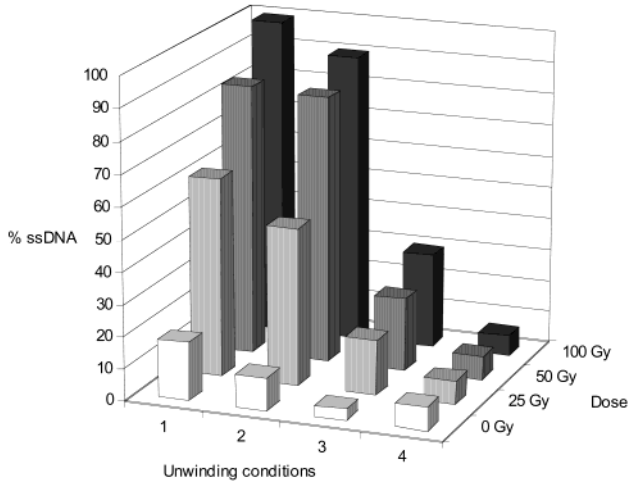


Figure 5. Percentage of ssDNA obtained with DNA isolated from sheep WBC as a function of both $\gamma$ radiation doses and unwinding conditions of (1) 0.05 M KOH and 10-min unwinding; (2) 0.05 M KOH and 5-min unwinding; (3) 0.01 M KOH and 20-min unwinding; (4) no unwinding.

Table 2. Calculated Yields of SB ($\times 10^6$ Base Pairs) Produced by $\gamma$ Radiation (in Gy), UV Light, and Intrinsic Causes in Living Mammals[a]

| discipline | SB | source of damage |
|---|---|---|
| radiotherapy treatments[22] | 0.371 | 2 Gy |
| chernobyl survivors[35] | 1.85 | 10 Gy |
| food irradiation[38−40] | 185 | 1000 Gy |
| drug phototoxicity studies[36,37] | 80−300[b] | 70 mJ/cm$^2$ (UV light) |
| spontaneous DNA damage[41] | 1.43 | metabolism, per hour |

[a] SB values are calculated by assuming 1 Gy of $\gamma$ radiation will form 1000 SSBs and 40 DSBs/cell (1 cell contains 6 pg DNA, which is equivalent to $5.61 \times 10^9$ base pairs). [b] Values are calculated from those previously reported, i.e., 0.5−2 SB for supercoiled $\Phi_\chi$-174 DNA (5386 base pairs).[36,37]

DNA damage studies performed in vitro to establish the phototoxic properties of pharmaceutical products are quantified in terms of SSBs produced. Phototoxic drugs reportedly produce ~1 SB/6000 base pairs.[36,37] With our technique, we are able to detect an even lower ratio of SB/base pair after appropriate unwinding of the DNA (see Table 2).

Food preservation is another discipline where $\gamma$ radiation of biological substrates is involved. Radiation doses in the order of 1 kGy or higher are usually employed in food preservation.[38−40] While the present conditions employed with this technique could not differentiate a $\gamma$ radiation dose of 100 from 1000 Gy, upon

(34) Pouget, J.-P.; Douki, T.; Richard, M.-J.; Cadet, J. *Chem. Res. Toxicol.* **2000**, *13*, 541−547.
(35) Gottlöber, P.; Steinert, M.; Weiss, M.; Bebeshko, V.; Belyi, D.; Nadejina, N.; Stefani, F. H.; Wagemaker, G.; Fliedner, T. M.; Peter, R. U. *Radiat. Res.* **2001**, *155*, 409−416.
(36) Artuso, T.; Bernadou, J.; Meunier, B.; Piette, J.; Paillous, N. *Photochem. Photobiol.* **1991**, *54*, 205−213.
(37) Chouini-Lalanne, N.; Defais, M.; Paillous, N. *Biochem. Pharmacol.* **1998**, *55*, 441−446.
(38) Beaulieu, M.; Béliveau, M.; D'Aprano, G.; Lacroix, M. *J. Agric. Food. Chem.* **1999**, *47*, 2537−2543.
(39) Zareena, A. V.; Variyar, P. S.; Gholap, A. S.; Bongirwar, D. R. *J. Agric. Food. Chem.* **2001**, *49*, 687−691.
(40) Fan, X.; Gates, R. A. *J. Agric. Food. Chem.* **2001**, *49*, 2422−2426.
(41) Billen, D. *Radiat. Res.* **1990**, *124*, 242−245.

proper adjustment of the unwinding conditions (reduced unwinding buffer concentration), our technique could rapidly assess whether food has been irradiated or not. Furthermore, it would be straightforward to establish whether foodstuff have been $\gamma$ irradiated, which is important in relation to regulatory compliance related to preservation and labeling.

The previous values and the corresponding disciplines are summarized in Table 2.

**Analyte Quantities.** Another important aspect involves the minimum concentration of analyte that can reliably be detected with our technique. Previous results show that high dye/DNA ratios (i.e., dye:DNA > 1:10) are not operational due to the possibility of intermolecular interactions between two dye molecules, which affect the kinetic laws employed in the fitting.[14] The dye concentration thus fixes that of DNA (i.e., DNA base pair molarity must be ~10-fold higher than the dye concentration). The minimum amount of dye (and therefore that of DNA) to be employed in our measurements is, on the other hand, predetermined by the sensitivity of our detector and the efficiency of the signal pickup of our optical setup.

The volume of sample irradiated by the laser, with each shot, is given by a cylinder of ~0.4 cm in length and ~0.4 cm in diameter (a volume of ~0.05 mL). Given that we can readily measure PicoGreen at concentrations of 10 $\mu$M when complexed with 100 $\mu$M DNA base pairs, it means that 0.5 nmol of dye and, correspondingly, 5 nmol of DNA base pairs can be easily detected with our system. This value is equivalent to ~3 $\mu$g of DNA.

Experiments performed with DNA embedded in gels produced 0.1-mL gels containing 6 $\mu$g of DNA each (i.e., DNA from $1 \times 10^6$ cells). These gels were then inserted into NMR tubes, where the sample was excited and a signal was measured (see Figure 1).

The wavelength limitations of the picosecond Nd:YAG laser allowed us to excite only at 355 nm. At this wavelength, the absorbance of PicoGreen is quite low, its molar extintion coefficient being 12.3 times lower than that at its absorbance maximum at 497 nm. In theory, using a 497-nm excitation wavelength would reduce the necessary amount of PicoGreen and, therefore, that of DNA by a factor of ~10. An improvement on the signal pickup would also result in a considerable decrease in the minimal amount of cells needed to assess DNA damage. Presently, only 6% of the fluorescence emission is picked up by a collimating lense of 3.6-cm aperture located 7.85 cm away from the sample. The use of an ellipsoidal mirror surrounding the sample would allow us to capture and measure over 80% of the emitted photons, enabling us to reduce the amount of cells needed for a detectable signal by an order of magnitude.

With these modifications, we foresee a reduction in the total amount of cells needed to detect a signal by a factor of ~100; that is, this technique could report DNA damage from a sample consisting of merely $10^4$ cells (equivalent to ~50 ng of DNA).

**Reproducibility.** All the results in Table 1 are the mean of three independent measurements while those obtained following doses of 5 and 10 Gy of $\gamma$ radiation (and also shown in Table 1) are the mean of six independent measurements. Since the standard deviation is maintained at ~5 in percentage of ssDNA, we can confirm that the results are reliable where differences in ratios of ssDNA to dsDNA are concerned.

It is important to mention that two main problems were encountered during the development of this technique. The first was the high fluorescence emission from the Gelbond film. In fact, the emission intensity from this material is as strong as that of the PicoGreen−dsDNA complexes that were used in our experiments. The second was that the fluorescence lifetime of the Gelbond film is in the range of those for PicoGreen−ssDNA and PicoGreen−dsDNA. Ultimately, we were able to overcome these problems by performing the lysing and unwinding steps with the agarose-embedded cells fixed to the Gelbond film; however, following the neutralization step, the gels are peeled from the Gelbond film and stained with PicoGreen, as summarized in Figure 1.

## CONCLUSIONS

We have introduced a new technique for DNA damage detection based on time-resolved fluorescence measurements of dye−DNA complexes. This technique is devoid of many of the problems encountered with other methods; thus, there is no need for operator judgment during the measurements of the sample, an issue encountered in the comet assay. In contrast with the currently employed steady-state fluorescence techniques,[11,13,22] there is no need for blanks given that the fluorescence lifetime is the measurement of the ratio of ssDNA to dsDNA. Furthermore, it is independent of the amount of sample employed, or its DNA content, provided that a ratio of <1:10 in dye to DNA base pairs is maintained in all the samples.[15]

At the present stage of development, this technique is able to detect DNA damage produced by a $\gamma$ radiation dose as low as 5 Gy and up to 100 Gy. We also believe that, upon appropriate conditioning, higher sensitivity could be achieved, allowing this technique to be used as a fast diagnostic tool to detect DNA damage from human exposure to low radiation doses.

Although the technique lacks the ability to monitor damage on a single-cell basis, such as in the comet assay,[6−9,42] its strength lies in the fact that one measurement encompasses the detection of a statistically relevant population of sample in a relatively short time. Yet, very little sample is necessary if modifications are made to our current setup; we estimate that as little as $10^4$ cells can be reliably measured at once.

Finally, we note that while our work involved a sophisticated picosecond fluorescence system, similar measurements could be developed around less expensive light sources, such as short pulse diodes.

## ACKNOWLEDGMENT

J.C.S. thanks the Natural Sciences and Engineering Research Council of Canada and the Canadian Space Agency for support. G.C. thanks the Ontario Graduate Scholarship Program for a postgraduate scholarship.

Received for review June 25, 2002. Accepted September 26, 2002.

AC025888J

(42) Böcker, W.; Rolf, W.; Bauch, T.; Müller, W.-U.; Streffer, C. *Cytometry* **1999**, *35*, 134−144.