DNA double-strand breaks as a method of radiation measurements for therapeutic beams

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Purpose: Many types of dosimeters are used to measure radiation dose and calibrate radiotherapy equipment, but none directly measure the biological effect of this dose. The purpose here is to create a dosimeter that can measure the probability of double-strand breaks (DSB) for DNA, which is directly related to the biological effect of radiation.

Methods: A DNA dosimeter, consisting of magnetic streptavidin beads attached to four kilobase pair DNA strands labeled with biotin and fluorescein amidite (FAM) on opposing ends, was suspended in phosphate-buffered saline (PBS). Fifty microliter samples were placed in plastic tubes inside a water tank setup and irradiated at the dose levels of 25, 50, 100, 150, and 200 Gy. After irradiation, the dosimeters were mechanically separated into beads (intact DNA) and supernatant (broken DNA/FAM) using a magnet. The fluorescence was read and the probability of DSB was calculated. This DNA dosimeter response was benchmarked against a Southern blot analysis technique for the measurement of DSB probability.

Results: For the DNA dosimeter, the probabilities of DSB at the dose levels of 25, 50, 100, 150, and 200 Gy were 0.043, 0.081, 0.149, 0.196, and 0.242, respectively, and the standard errors of the mean were 0.002, 0.003, 0.006, 0.005, and 0.011, respectively. For the Southern blot method, the probabilities of DSB at the dose levels of 25, 50, 100, 150, and 200 Gy were 0.053, 0.105, 0.198, 0.235, and 0.264, respectively, and the standard errors of the mean were 0.013, 0.024, 0.040, 0.044, and 0.063, respectively.

Conclusions: A DNA dosimeter can accurately determine the probability of DNA double-strand break (DSB), one of the most toxic effects of radiotherapy, for absorbed radiation doses from 25 to 200 Gy. This is an important step in demonstrating the viability of DNA dosimeters as a measurement technique for radiation. © 2018 American Association of Physicists in Medicine [<https://doi.org/10.1002/mp.12956>]

Key words: DNA dosimeter, radiation dosimetry, radiation therapy measurements, Southern blot analysis

1. INTRODUCTION

Radiation is one of the most powerful tools available for treating cancer. Its therapeutic effect results from the biological damage it produces. This damage is capable of disease control, but can also create toxicity to the surrounding normal tissue. For each patient case, physicians carefully balance the potential for both when prescribing a course of radiotherapy. It is important that they not only prescribe the optimal amount of radiation but also that the radiotherapy equipment is accurately characterized and calibrated to deliver this optimal amount. Although biological damage is the end goal for radiotherapy, the methods utilized to calibrate the equipment are not biologically based, which poses a potential knowledge gap for the effect of delivered radiation. The long-term hypothesis of this work is that biological-based dose measurement devices (dosimeters) will enable more accurate and meaningful characterizations and calibrations of radiotherapy equipment.

The goal for accuracy of radiotherapy is set in accordance with the International Commission on Radiation Units and Measurements (ICRU) Report 24, which states that treatments are expected to deliver within 5% of the intended dose, when accounting for all sources of uncertainty, to prevent poor clinical outcomes.¹ Linear accelerators (LINACs) are the most common method for delivering radiotherapy. These machines produce, shape, and target radiation beams to tumors, while minimizing the impact to surrounding normal tissue. The radiation type and energy, size of the collimated shape, depth in the patient, and tissue density heterogeneities all affect the radiation transport and energy deposition. For this reason, behind every LINAC is a beam model, which represents how these changes affect energy deposition. These models are ultimately built on a collection of radiation measurements made in tissue-equivalent materials. Inaccuracies in any aspect of these measurements yield flaws in the beam models, which then translate to errors in radiation delivery to patients.

The potential knowledge gap for radiation measurements has two aspects to it. The first part of this concerns the relationship between radiation prescriptions and measurements. These prescriptions are written in units of absorbed dose to tissue, which represents the amount of radiation energy absorbed per unit mass. Clinically though, absorbed dose is impractical to directly measure. Instead, calibration protocols utilize charge measurements from ionization chambers, along with beam- and chamber-specific correction factors, to determine the absorbed radiation dose.² There are several clinical scenarios where the ability to connect charge measurements to absorbed dose becomes difficult: small radiation treatment fields, shallow treatment depths, and regions of tissue inhomogeneity.^{3–7} The uncertainty in this connection alone can approach the overall 5% treatment accuracy goal. The second part of the knowledge gap, which is relevant to this work, is that absorbed dose does not serve as a perfect proxy for biological damage, since different types of radiation, at the same absorbed dose, yield different levels of biological damage.⁸ There are assumed radiation-weighting biological effectiveness factors that are meant to account for these dependencies, but there is a significant amount of uncertainty associated with these factors.⁹

A potential solution to the biological knowledge gap is to create dosimeters that directly measure biological damage. While there are many factors that impact an individual cell's death, such as damage to cellular organelles and the bystander effect, DNA DSBs are generally accepted to be the dominant factor for ionizing radiation-induced cell killing because even a single unrepaired one can cause cell death.^{10–13} Although DSBs may not provide a full picture of cell killing, a dosimeter that uses DSBs as a dose metric could circumvent the biological knowledge gap for radiation measurements and provide a more meaningful representation of radiation-induced biological damage.

Most of the previous work on DNA dosimetry focused on evaluating DNA base damage.^{14–22} However, there was one previous work that attempted to directly measure DSB for dosimetry.²³ In brief, they used circular DNA as their dosimeter. A DSB would cause the circular DNA to become linear. Then, gel electrophoresis was used to separate circular from linear DNA to find the probability for DSB. Their work showed great promise, but at that point, it was not accurate or practical enough to be used routinely in radiotherapy clinic. It has been 20 yr since this work was published, without any follow-up.

Advances in molecular medicine and cellular and structural biology, such as the use of fluorescent tagging, fluorescence detection, and streptavidin beads, have not yet been applied to radiation dosimetry. These advances were harnessed to create a DNA DSB dosimeter. To best reflect *in vivo* DSBs, the dosimeter should be irradiated in a solution that is similar to cells. For the gel electrophoresis method, a user would need to irradiate the dosimeter in one solution, transfer to another, and then they would need to wait for the gel separation. Our technique is able to separate the broken from unbroken DNA in the same solution it is irradiated in,

in about a minute. Although it is not currently done in this way, this could enable a DNA dosimeter to be irradiated, separated, and read all in the same vessel, in a matter of minutes, which is the main advantage that our technique has over the gel electrophoresis method. This manuscript describes the proof-of-principle evaluation of this dosimeter response and benchmarking of it against Southern blot analysis.

2. MATERIALS AND METHODS

2.A. The DNA dosimeter

The DNA dosimeter consists of double-stranded DNA (dsDNA) tagged with fluorescein amidite (6FAM) and biotin, along with magnetic streptavidin beads. Figure 1(a) shows the dosimeter before it is irradiated. The biotin DNA end binds to the streptavidin bead. These strands are suspended in phosphate-buffered saline (PBS) to form the usable dosimeter. Figure 1(b) displays the dosimeter as x-ray radiation interacts with the DNA strands. In the case of a DSB, the strands will be broken into two pieces: the bead/biotin end and the 6FAM end (fluorescing broken ends). Figure 1(c) shows a magnet being used to physically separate the unbroken pieces from the broken pieces. The magnet attracts the beads and holds the intact dsDNA as well as nonfluorescent, broken dsDNA. The solution that remains, called the supernatant, is then extracted. The remaining beads are then resuspended in an equal volume of PBS. These samples are plated and read on a fluorescence reader. The relative fluorescence of the broken and unbroken dsDNA is utilized to determine the probability for DSB.

FIG. 1. (a) The pre-irradiated dosimeter, (b) x-ray radiation interacting with the dosimeter causing double-strand breaks, and (c) the separation of broken DNA. [Color figure can be viewed at wileyonlinelibrary.com]

2.B. Dosimeter fabrication

The first step of the dosimeter fabrication was a polymerase chain reaction (PCR) to create the desired strand length of DNA. A pRS-316 vector was used as the template for this. Biotin-labeled and 6FAM-labeled oligonucleotides of 24 base pair sequences were used as primers for the PCR. This fluorescein label has an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The biotin-labeled oligonucleotide sequence used was: 5'-ggcagcactgcataattcttactg -3' (pRS-316 sites 3802-3827). The 6FAM-labeled oligonucleotide sequence was 5'-atcaagagctaccaactcttttccg-3' (pRS-316 sites 3037-3062). The expected strand length was 4147 base pairs. The end PCR product is a dsDNA fragment with biotin on one end and 6FAM on the other.

Before attaching streptavidin beads to the PCR product, the length of the dsDNA PCR product was verified against DNA ladder markers using standard gel electrophoresis. After length verification, the 4 kb pair dsDNA were attached to Dynabeads MyOne streptavidin T1 beads (Thermo Fisher Scientific, Waltham, MA) through an incubation process using binding buffer. The biotin end connects the dsDNA to the streptavidin bead. After attachment, these bead-connected dsDNA were suspended in a washing buffer solution (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2.0 M NaCl) for storage. The bead to dsDNA mass ratio was varied to optimize the cost of the dosimeter. At the optimal cost, the binding efficiency of the dsDNA to the beads was on the order of 30%. All unattached strands are separated and removed from the samples after the attachment process.

2.C. Sample irradiation

Five separate experiments were performed in which DNA dosimeter samples were irradiated with 6 MV photons from a Clinac 600 C/D linear accelerator (Varian, Palo Alto, CA) to 25, 50, 100, 150, and 200 Gray (Gy). For these irradiations, 1000 μ L of dosimeter solution was extracted from the same batch on the morning of the irradiation. The samples were stored in a washing buffer to preserve them. Before usage, they were washed and resuspended in 1000 μ L of phosphate-buffered saline (PBS). Then, 50 μ L aliquots of the bead-connected dosimeter, in which each contains 4.2 μ g of dsDNA, were placed in 1.5 mL microcentrifuge tubes for irradiation.

During irradiation, the dosimeters were immersed within 1.5 mL microcentrifuge tubes inside a water tank (MP3, PTW, Freiburg, Germany) at a 5 cm depth and at 65 cm from the radiation source. For each experiment, three dosimeters were irradiated at each dose level, plus three dosimeters for background count (0 Gy). A total of 18 dosimeters were used per experiment and 90 dosimeters total. A Southern blot analysis was performed on the supernatant from each of the samples for three of the total five experiments (54 total dosimeters). Before irradiating the dosimeters, a calibrated 0.3 cm³ Semiflex 31013 ionization chamber (PTW, Freiburg,

Germany) was utilized along with the established calibration protocols¹ to verify the dose levels.

2.D. Dose response: fluorescence measurement

After irradiation, a DynaMag magnet was used to separate the supernatant from the beads. Samples were plated and the fluorescence intensity read with a Synergy2 fluorescence reader (BioTek Instruments Inc, Winooski, VT). The probability of DSB for the DNA strands was calculated as the number of broken strands divided by the total number of strands. Thus, this represents the probability of one or more DSBs for the 4 kb DNA strands. Corrections were made to the fluorescence readings of the supernatant and beads (F_s and F_B , respectively) to obtain values that are representative for the number of the broken and total DNA strands. First, a dark count was removed from each of fluorescence readings. For the supernatant, the fluorescence reading for 50 μ L volumes of pure PBS (F_P) was served as the dark count. The beads themselves fluoresce, so the background dark count for the beads was from the reading of 50 μ L volumes of PBS with same concentration of beads as that in the dosimeter (F_{BB}). One additional correction was made to account for the opacity that the beads introduce when suspended. For this, the fluorescence was read from free 6FAM at a constant, known concentration similar to our dosimeter suspended in 50 μ L volumes of both pure PBS and in PBS with same concentration of beads as that in the dosimeter (F_{PFAM} and F_{BBFAM} , respectively). The fluorescence readings from these were utilized to characterize an attenuation factor (A). This factor represents the ratio of the amount of light that would be collected in the absence of the bead opacity:

$$\text{Attenuation Factor} = A = \frac{F_{PFAM} - F_P}{F_{BBFAM} - F_{BB}} \quad (1)$$

Then, the probability for DNA double-strand break (P_{DSB}) was calculated with the following equation.

$$P_{DSB} = \frac{F_s - F_P}{F_s - F_P + A \cdot (F_B - F_{BB})} \quad (2)$$

The measured P_{DSB} for the nonirradiated samples was utilized as a dark count and was subtracted from that of the irradiated samples.

2.E. Dose response: Southern blot visual confirmation and quantification

After fluorescence reading, the samples were transferred back to tubes for the gel electrophoresis and subsequent Southern blot analysis. A set of supernatant samples at each dose level was run on a 0.7% agarose gel to separate the DNA strands by size. After running on the gel, DNA was transferred onto an Amersham Hybond-N membrane (GE Healthcare, Little Chalfont, UK) by the capillary transfer method. DNA was cross-linked onto the membrane by UV and hybridization with a 5' end labeled with γ -32P-ATP

(sequence: atcaagagctaccaactcttttccgaaggtaactggcttcagcagagcg). The probe was detected by using a Typhoon FLA7000 phosphor imaging machine (GE Healthcare, Little Chalfont, UK), and analyzed using ImageQuant 5.1. In addition to the supernatant, a PCR-only input and empty lane were both run. The PCR-only input had the same total number of 4 kb DNA strands as a 50 μ l dosimeter, and the empty lane was utilized as a background count. Equation (3) displays the calculation of the probability for DNA DSB from these lane intensities.

$$P_{DSB} = \frac{I_S - I_E}{I_{PCR} - I_E} \quad (3)$$

The quantity I_S is the intensity of the supernatant at each dose level, I_E is the intensity of the empty lane, and I_{PCR} is the intensity of the PCR-only input. The radiolabeling of the DNA occurs at the same strand end as that of the fluorescein. Thus, after background subtraction, the ratio of the supernatant to the PCR-only input represents the fraction of the DNA strands with a DSB.

2.F. The response accuracy of the DNA dosimeter

The mathematical metric we used to quantify this dose measurement accuracy is the coefficient of variation (COV), which is the ratio of the standard deviation to the mean reading. A lower value of COV indicates less variation in response, which means a more precise response of the DNA dosimeter. The ultimate utility of the DNA dosimeter is to make dose measurements and COV is closely related to how precisely we can make measurements with this dosimeter.

3. RESULTS

Figure 2 shows a digitized phosphor image for the position and relative quantity of P^{32} -labeled DNA strands from the supernatant for nonirradiated and irradiated DNA dosimeters, compared to a DNA ladder. The nonirradiated, preattachment “PCR only” lane shows a defined line at 4 kbp (kbp), indicating that the DNA present is intact. The 0–200 Gy supernatant lanes show an increase in the darkening

FIG. 2. Digitized phosphor image of the Southern blot DNA analysis. The DNA ladder marker lengths greater than 3 kbp were not clearly separated from each other.

FIG. 3. Plot of double-strand break probability for each method of analysis. The error bars represent the standard error of the mean. [Color figure can be viewed at wileyonlinelibrary.com]

of the lane with an increase in dose, indicating that we are measuring DNA breakage and not a degradation of the dosimeter components. Additionally, the lane shows a vertical smear pattern, indicating a variety of DNA strand sizes exist. The absence of any distinct bands within the lanes means that the damage is random and nonspecific to a location on the DNA strand.

Experiments 1, 2, and 3 occurred with both the DNA dosimeter and Southern blot analysis. Experiments 4 and 5 only utilized the DNA dosimeter. Figure 3 displays the plot of the measured probability of DNA DSB with the corresponding standard errors of the mean plotted as errors bars for each method of analysis (for Experiments 1, 2, and 3). Table I shows the corresponding average data for each dose level and method of analysis. Table II displays the achieved DNA dosimeter COV at each dose level for all five experiments.

TABLE I. Probability of double-strand breaks for each method of analysis along with the standard error of the mean.

Dose (Gy)	Fluorescence intensity corrected		Southern blot quantification	
	Probability of DSB	$s/\sqrt{n}$	Probability of DSB	$s/\sqrt{n}$
25	0.043	0.002	0.053	
50	0.081	0.003	0.105	0.024
100	0.149	0.006	0.198	0.040
150	0.196	0.005	0.235	0.044
200	0.242	0.011	0.264	0.063

TABLE II. The COV for the probability of DSB achieved for each experiment and dose.

Dose (Gy)	Experiment #				
	1	2	3	4	5
25	0.129	0.030	0.138	0.030	0.026
50	0.040	0.123	0.087	0.045	0.019
100	0.042	0.037	0.131	0.031	0.033
150	0.057	0.071	0.045	0.008	0.011
200	0.065	0.065	0.047	0.010	0.006

4. DISCUSSION

The data displayed in Fig. 3 and Table I show that the DNA dosimeter can successfully measure DSB as a function of ionizing radiation dose. An important additional aspect about the dosimeters is how precisely they can measure dose. Since our overall goal is to deliver dose to patients within 5% of that intended, when including all sources of uncertainty, our dosimeters would ideally produce dose accuracy that is much better than this. A COV of 1% is similar to that which can be obtained from other dosimeters used in radiation measurements: optically stimulated luminescent dosimeters (OSLDs),²⁴ thermoluminescence dosimeters (TLDs), and film. The data in Table II demonstrate that COV of 1% is achievable with the dosimeter. However, these are achieved at doses which are much higher than the typical patient dose per fraction. These COV values increase with lower doses. As an example, previous experiments achieved COV of 14% and 7% at the doses of 5 and 10 Gy, respectively.²⁵ With the dosimeter's current sensitivity, its only direct application would be measurements for stereotactic radiosurgery, where doses extend into the range of the dosimeter. In terms of ways to improve the dosimeter sensitivity, the best way would be to increase the length of the attached DNA strands. In a first-order approximation, the dose required to produce a certain P_{DSB} would be inversely proportional to the length of the DNA. Thus, if we were to attach a strand with an order of magnitude longer length, it should be sensitive to an order of magnitude lower doses.

These data show a large variability in the COV. For example, at the lowest dose, COV around 3% are found but also one that is almost 14%. Thus, further refinements would certainly be necessary before these would be directly useful for clinical radiotherapy measurements. The current sample analysis process is still quite manual, requiring hours of pipetting per experiment, which makes it prone to human fatigue and variabilities in sample collection. We believe this to be a significant factor leading to the large variability in the COV and expect that automated systems for sample collection and separation would improve this variability. Beyond working to improve the sensitivity and variability of the dosimeter, future work will focus on comparing the dosimeter response to DNA Monte Carlo simulations^{26–29} and *in vitro* cell irradiation as well as the measurements of small-field output factors. DNA Monte Carlo can be compared directly to the dosimeter response. For cell comparison, we aim to make relative biological effectiveness measurements with the dosimeter and compare it to that measured with cell lines, irradiated under the same conditions. Small-field output factors measurements will be compared to those made by small-field dosimeters and corrections thereof.³⁰

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