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Short communication

Oligonucleotide chip assay for quantification of gamma ray-induced single strand breaks

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ABSTRACT

An oligonucleotide chip assay was designed for direct quantification of single strand breaks (SSBs) induced by γ-ray irradiation. The oligonucleotides used were 20-mers, which were short enough to produce only a single strand break within a single oligonucleotide. The two ends of the oligonucleotides were labeled with fluorescein and biotin, respectively. The biotinylated ends of the oligonucleotides were immobilized on a silicon wafer chip treated with (3-aminopropyl)triethoxysilane (APTES), glutaraldehyde, and avidin. The DNA fragments cleaved by γ-ray irradiation were detected by a laser-induced fluorescence (LIF) detection system. The γ-ray-induced SSBs were quantified using a calibration curve (fluorescence intensity versus γ-ray dose) without the need for complicated mathematical calculation based on gel-based separation. The experimentally determined γ-ray-induced SSBs yield was almost equal to the theoretical value derived from gel electrophoresis of plasmid DNAs and DNA surface coverage.

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1. Introduction

DNA microarray is based on hybridization of a target DNA to a probe DNA that is immobilized on a solid support [1–3]; the probe DNA and target DNA are designed such that they share complementary DNA sequences. This hybridization technique has been actively used in numerous biological applications such as biomarker discovery [4,5] or disease diagnosis [6–8]. DNA fragmentation is another attractive assay for DNA microarray: exposing the immobilized DNA to a toxic chemical can result in the cleavage of DNA immobilized on the solid support. Photo and drug-induced genotoxicity measurement using a DNA chip is an appropriate example [9,10]. Ionizing radiation-induced radical products such as hydroxyl radicals are another stimulus that causes strand breaks in DNA [11].

The goal of radiotherapy in treating cancer patients is to kill all of the malignant cells within a tumor or to lessen symptoms by reducing local tumor burden. In radiotherapy, the total dose of radiation is an important issue because the administered radiation can cause toxicity; the tolerance of normal tissue within the radiation field is also limited by radiation dose. Recent advances in molecular biology have revealed that ionizing radiation has diverse effects on cellular functions. Radiation can affect cell membranes and organelles [12]. DNA is a critical target of radiation therapy and unrepaired DNA strand breaks lead to chromosomal aberrations and cell death following subsequent mitosis [13]. There are several quantitative assays for determining the extent of DNA damage induced by ionizing radiation, such as intact band depletion assay [14], fraction of activity released (FAR) assays [15], comet assays [16–18], and clustered DNA damage assays involving gel electrophoresis and enzyme reactions [19,20]. A common feature of these assays is DNA fragment separation using gel electrophoresis and subsequent DNA size analysis on the gel using a theoretical or empirical formula.

In this study, we explored the possibility of quantitatively determining γ-ray-induced DNA single strand breaks (SSBs) using a short single-stranded oligonucleotide microarray immobilized on silicon wafer. Considering the fact that the average number of γ-ray-induced SSBs is 55–67 SSBs per megabase per Gy [19,21], it can be assumed that the possibility of more than one single strand breaks in an oligonucleotide probe as short as 20-mer is negligible. Therefore, if immobilized fluorophore-labeled oligonucleotides are exposed to γ-ray radiation, the amount of oligonucleotides in the supernatant theoretically represents the total number of single strand breaks. Unlike in gel electrophoresis-based quantification, DNA fragments released from the silicon wafer by γ-ray are not subject to the restraint of their separation for quantitative analysis in DNA microarray. This study indicates that the DNA fragmentation assay using a DNA microarray is a promising technique for quantitatively measuring SSBs caused by γ-rays.

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2. Materials and methods

2.1. Reagents

(3-Aminopropyl)triethoxysilane (APTES), toluene, ethylenediamine tetraacetic acid, disodium salt (EDTA), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), Tris–HCl aqueous solution (1 M), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), avidin, and alkaline phosphatase were purchased from Sigma (St. Louis, MO). Glutaraldehyde was obtained from Electron Microscopy Sciences (Fort Washington, PA). Silicon wafers coated with a 1-μm thick oxide layer were received from Celltek Co., Ltd (Gyeonggi Technopark, Ansan-si, South Korea). The custom-built 5′-fluorescein-labeled and 3′-biotinylated oligonucleotide was purchased from GeneChem Inc. (Daejeon, South Korea). The oligonucleotide sequence was 5′-FAM-CTTCCTCTGTAGCTTGCTCT-Biotin-3′, which corresponds to the conserved region of the HIV gag gene. Autoclaved, purified water (18.0 MΩ) was used for the aqueous buffer media for the oligonucleotides.

2.2. Calibration curve of fluorescence intensity versus oligonucleotide concentration

The calibration curve of fluorescence intensity versus oligonucleotide concentration was measured using a laser-induced fluorescence (LIF) detection system. The LIF detection system was previously described in detail [22]. The 5′-fluorescein-labeled and 3′-biotinylated oligonucleotide stock solution (1×10^{-4} M) was serially diluted with buffer to prepare a series of sample solutions with concentration ranging from 1×10^{-9} M to 1×10^{-7} M. The buffer solution was composed of 50 mM Tris, 250 mM sodium chloride, and 1 mM magnesium chloride. The sample solutions were injected into the capillary sample container. Laser-induced fluorescence was collected using a microscope objective lens and focused onto a photomultiplier tube (PMT). Signals from the PMT were read and saved in a laptop computer using custom software and interface systems. The pH of the sample solutions was adjusted to 9.9 before obtaining the calibration curve used for DNA surface coverage determination. On the other hand, when the number of SSBs induced by γ-ray was determined, the pH of sample solutions to acquire the calibration curve was adjusted to 8.0.

2.3. Fabrication of oligonucleotide chip

Fig. 1 illustrates the oligonucleotide chip fabrication process. Silicon oxide wafers (10 mm × 6 mm) were washed sequentially with chloroform, isopropyl alcohol, methanol, and water and then dried with N_2 gas. The cleaned wafers chips were silanized in 5% (v/v) APTES in dry toluene by heating overnight at 115 °C. The unbound silane was removed by sonicating the chips in toluene. Subsequently, the chip was activated with 5% (v/v) glutaraldehyde in potassium phosphate buffer (10 mM, pH 7.0) for 4 h at room temperature, and then washed with potassium phosphate buffer to removed unbound glutaraldehyde. Chip was then treated with avidin dissolved in potassium phosphate buffer (10 mM, pH 7.0). Fluorescein-biotinylated 20-mer oligonucleotides were finally attached to the chip surface via strong avidin-biotin interaction by immersing the chips in aqueous oligonucleotide immobilization solutions (final oligonucleotide concentration of 3 μM) at 4 °C for 48 h. The immobilization solution was made in 10 mM potassium phosphate buffer (pH 7.0) containing 0.3 M NaCl. Following oligonucleotide deposition, the films were rinsed with buffers and stored in buffer solution.

2.4. Determination of DNA surface coverage

The total number of oligonucleotides immobilized on the chip was determined by alkaline phosphatase (ALP) assay. ALP was dissolved in buffer solution (50 mM Tris, 250 mM sodium chloride, and 1 mM magnesium chloride) and its final concentration was adjusted to 1.9 units/mL. The oligonucleotide chip was immersed in ALP solution (pH 9.9) for 2 h at 37 °C. The cleaved single strand DNA (ssDNA) was released into the buffer solution. The chip was then removed from the buffer solution. The cleaved ssDNAs were collected and their fluorescence intensities were measured using the LIF detection system. The fluorescence originated from the fluorescein tag at the end of the cleaved ssDNAs. The fluorescence intensity was substituted into the empirical formula of the calibration curve acquired at pH 9.9 and the corresponding concentration was selected. The

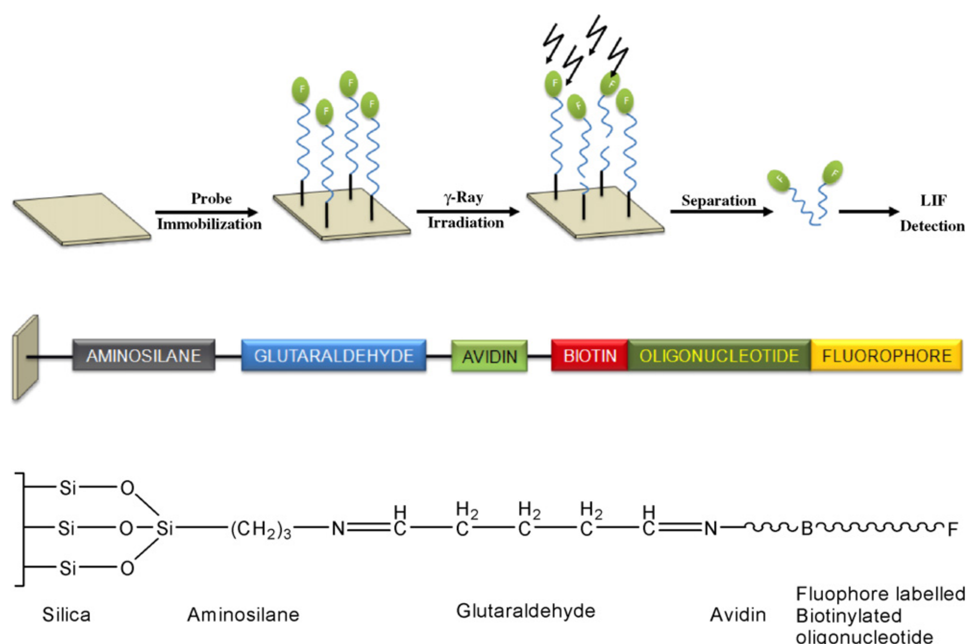

Fig. 1. Schematic diagram of oligonucleotide chip fabrication.

total number of DNA molecules was determined by multiplying the corresponding concentration by the buffer volume immersing the chip. The total number of DNA molecules was divided by the surface area of the chip to acquire DNA surface coverage.

2.5. γ-Ray dose-dependent ssDNA strand breaks

The oligonucleotide chip was placed on the bottom of a polycarbonate chamber (1 cm × 1 cm × 1 cm) filled with 150 μL of potassium phosphate buffer (pH 8.0; 50 mM Tris, 250 mM sodium chloride, and 1 mM magnesium chloride). The cover of the polycarbonate chamber was opened so that ^{137}Cs γ-ray could reach oligonucleotides immobilized on the chip during irradiation. The polycarbonate chamber was placed on ice during γ-ray irradiation. The ^{137}Cs γ-ray irradiation was carried out using a gammacell low dose-rate research irradiator (Model #: GC 3000 Elan, MDS Nordion, Canada) in National Center for Inter-University Research Facilities (NCIRF) at Seoul National University. The irradiation doses of ^{137}Cs γ-ray were 5, 20, 40, and 80 Gy at a dose rate of 3.5 Gy/min. After irradiation, the immersing buffer solutions containing fluorescein-tagged oligonucleotide fragments were separated from the chips and analyzed using the LIF system. In order to quantify the number of SSBs induced by γ-ray as a function of dose, the fluorescence intensity was substituted into the empirical formula derived from a calibration curve obtained with standard sample solutions at pH 8.0.

3. Results

3.1. Calibration measurement of fluorophore-labeled oligonucleotides

In order to determine the number of DNA fragments cleaved by γ-ray irradiation, a calibration curve (slope: 0.999; linear regression coefficient: 0.986) of fluorescence intensity versus fluorescein-labeled oligonucleotide concentration was obtained (Fig. 2). The detection limit at S/N = 3 was 3.0×10^{-10} M. This value is indicative of the minimally detectable amount of oligonucleotide immobilized on the chip. In other words, 4.5×10^{-14} mol of cleaved oligonucleotides can be detected in 150 μL of immersion buffer solution over the chip. Assuming that the chip was fully covered with two-dimensional oligonucleotide-monolayers of density 6 pmol/cm^2, this approach can detect a submonolayer lower than 0.75% if the functionalized surface area is ≥1.0 cm^2 and the volume of the solution is ≤150 μL.

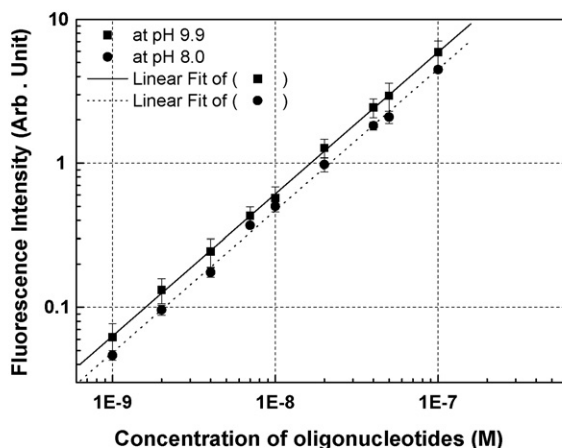

Fig. 2. Fluorescence intensities of fluorescein-biotinylated oligonucleotides as a function of concentration. The upper linear curve (Slope: 0.985, Regression coefficient: 0.986) was obtained at pH 9.9, while the lower linear curve (Slope: 0.985, Regression coefficient: 0.986) was obtained at pH 8.0. The excitation and emission wavelengths were 492 nm and 520 nm, respectively.

3.2. DNA surface coverage determination

An alkaline phosphatase (ALP) assay was performed to determine the DNA surface coverage of the oligonucleotide chip. The silicon wafer was treated with APTES in toluene and with glutaraldehyde in potassium phosphate buffer, followed by avidin (Fig. 1). The biotinylated oligonucleotides were then immobilized on the silicon wafer and treated with ALP solution. ALP treatment causes single-strand breaks at the phosphodiester backbone of the DNA on the silicon wafer. The cleaved fluorescein-labeled DNA fragments were then released into the buffer solution. The LIF was measured following injection of the solution into the capillary reactor. The cleaved DNA concentration (M) was calculated by substitution of the LIF signal into the mathematical formula derived from the calibration curve. The cleaved DNA concentration was then multiplied by the reaction volume (600 μL) and Avogadro's number, and divided by the surface area of the chip (1.2 cm^2). The average number of oligonucleotides immobilized on the chip was $3.55 \pm 0.06 \times 10^{12}$ molecules/cm^2 (5.90 ± 1.09 pmol/cm^2).

3.3. Formation of SSBs by ^{137}Cs γ-ray irradiation

When γ-radiation is absorbed in aqueous solution covering an oligonucleotide chip, radical species such as ·OH radicals, solvated electrons, and ·H atoms, are produced around the oligonucleotides. Among these radical species, solvated electrons do not induce DNA strand breaks. On the other hand, ·OH radical can induce DNA strand breaks by cleaving the phosphate-ester bond in DNA without modifying the DNA. The fluorescein-labeled ssDNA fragments were measured quantitatively as a function of ^{137}Cs γ-ray dose. To minimize unintended DNA damage by heat during γ-ray exposure, the polycarbonate reaction chamber was placed on ice. The method for determining the number of γ-ray induced cleaved DNA fragments was the same as that for determining DNA surface coverage. Fig. 3 shows the effects of γ-ray dose on SSB production. Table 1 compares the experimental SSB values determined using oligonucleotide chips with those predicted based on DNA surface coverage. At various doses, the number of experimentally obtained SSBs was very similar to the predicted values within experimental error. The number of SSB cleavages was calculated to be 71 SSBs per megabase per Gy, which was close to the 57–67 SSBs per megabase per Gy obtained in other investigations.

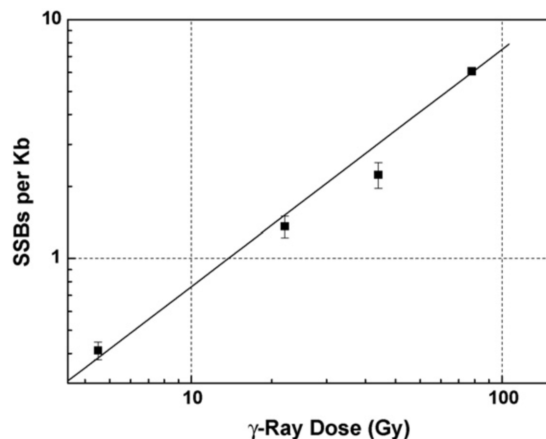

Fig. 3. A plot of SSBs induced by γ-ray as a function of γ-ray dose (Slope: 0.993, Regression coefficient: 0.997). From the slope, a quantitative value of 71 SSBs per megabase (Mb) per Gy was obtained.

Table 1
Comparison of single-strand breaks determined experimentally (SSBs) using the oligonucleotide chip with SSBs calculated theoretically based on DNA surface coverage and assuming 66 SSBs per Mb per Gy.

γ-Ray dose (Gy)	SSBs (per cm^2)	
	Experimental values ($\times 10^{10}$)	Theoretical values ($\times 10^{10}$)
5	2.98 ± 0.21	2.35
20	9.65 ± 0.95	9.40
40	16.82 ± 3.99	18.80
80	39.52 ± 12.74	37.59

4. Discussion

In this study, a short-length oligonucleotide chip assay was explored as a novel approach for quantitatively measuring DNA strand breaks caused by ionizing radiation. The procedure for determining SSBs using the oligonucleotide chip consisted of fabricating the oligonucleotide chip, establishing a calibration curve, and calculating the number of SSBs based on a mathematical formula. 20-mer oligonucleotides were covalently bound to a silicon wafer that was chemically treated with APTES and glutaraldehyde. A primary amine group of APTES bound on the silicon wafer reacts with the aldehyde group of glutaraldehyde and forms an imine linkage. Finally, strong conjugations form between the biotin label on the oligonucleotides and the avidin coated on the chip.

This DNA immobilization technique provides a unique environment for quantitatively determining γ-ray-induced SSBs. Only DNA fragments cleaved by γ-rays are released into the immersing buffer solution because one end of the oligonucleotide is fixed on the surface of the silicon wafer through the strong affinity between biotin and avidin. Furthermore, the cleaved oligonucleotides contain a fluorescein label. Therefore a cleaved oligonucleotide fragment corresponds to a SSB induced by γ-ray, unless multiple strand breaks have occurred within an immobilized oligonucleotide. In phosphate buffer, Milligan et al. observed 66 SSBs per Mb per Gy [21]. Based on this value, the probability of γ-rays inducing more than 2 SSBs within a 20-mer oligonucleotide is low. Therefore, we used a short-length oligonucleotide in the present study. This approach allowed direct quantitative measurement of γ-ray-induced SSBs on the DNA chip and eliminated the need to separate cleaved DNA fragments to measure single strand breaks because the number of single strand breaks was not dependent on the size of the cleaved DNA fragment on the DNA chip.

Acrylamide sequencing gels [23], agarose gel electrophoresis [24] and field direction gel electrophoresis [25] have been the most general separation techniques for determining SSBs. Intact band depletion has a strong mathematical foundation that allows for detection of DNA strand breaks and damage sites. The fraction of DNA that remains unbroken following enzymatic treatment is determined by gel electrophoresis, which separates the restricted DNA fragments from unbroken intact DNA. The zeroth poission probability coefficient gives the fraction of unbroken DNA following enzymatic treatment. However, safety problems arise from the use of radionucleotide labels in intact band depletion assays. The detection sensitivity is another inevitable limitation of the assay. Only when the length of intact DNA is appropriately large can the zeroth poission probability coefficient be measured. This phenomenon fundamentally limits the size of intact DNA that can be analyzed. Compared to intact band depletion, the DNA chip assay is much less restrictive in terms of the size of DNA that can be tested. Using a smaller DNA minimizes the possibility of multiple strand breaks occurring within the immobilized DNA. Fraction of activity released (FAR) assays and comet assays are also widely used to assess DNA damage. Unlike intact band depletion, these assays do not involve quantitative theory for measuring damage frequencies and the fraction of damaged DNA. Rather, empirical calibrations are used for quantitative measurements. Although these assays have their own advantages, they still require time-consuming gel electrophoresis.

The number of SSBs obtained by DNA microarray was compared with that measured by other research groups in order to validate the accuracy of the DNA fragmentation assay in the present study. This comparison was performed with doses ranging from 5 Gy to 80 Gy, as shown in Table 1. Under conditions without any radical scavengers, Milligan *et al.* observed 66 SSBs per Mb per Gy in sodium phosphate (pH 7.0). Similarly, Brake [26] and Sutherland et al. [19] observed 55–67 SSBs per Mb per Gy and 57 SSBs per Mb per Gy, respectively, in phosphate buffer. Of these 3 similar SSBs values, 66 SSBs per Mb per Gy was selected to represent theoretical SSBs and was compared with SSBs acquired experimentally in the present study. To obtain the theoretical SSBs, the total oligonucleotide size on the DNA microarray was multiplied by 66 SSBs per Mb per Gy at each dose. The total oligonucleotide size was determined from the DNA surface coverage. The number of immobilized oligonucleotides was multiplied by 20-mer to derive the total oligonucleotide size. At each dose, the correlation coefficients between the theoretical and experimentally obtained SSBs values were 0.99 and were within experimental error. The SSBs induced by γ-ray from 5 Gy to 80 Gy was 71 SSBs per megabase (Mb) per Gy according to this short-length oligonucleotide chip assay, as shown in Fig. 3. This corresponds to one SSB within 704 ssDNAs molecules at a 1-Gy dose.

It is noteworthy that at relatively higher dose a saturation effect could be expected between the number of broken 20-mer molecules and the dose. For example, after a substantial portion of the immobilized probes are broken, only a part of the incoming radiation will be utilized to break the immobilized probes and release the fluorophore-labeled fragments, whereas the rest of the incoming radiation will cause breakage in the fragmented oligonucleotides. However, we did not observe this effect in the studied range of dose (5–80 Gy). While this cannot be explained unambiguously with the present data set, we may speculate that the possibility of incoming radiation to further induce SSB in the already broken 20-mer probe, which is even shorter than the parent molecule, is almost negligible. However, this possibility of saturation effect cannot be completely ruled out particularly at an even higher dose and especially if the fragmented probe size is sufficiently longer.

5. Conclusion

A rapid, simple and convenient DNA fragmentation assay using an oligonucleotide chip was developed to quantify γ-ray-induced SSBs. This short-length oligonucleotide chip assay has potential as an alternative to gel-based quantification of SSBs induced by γ-ray. The SSB values obtained from the oligonucleotide chip assay were almost identical to those predicted from gel electrophoresis-based assays, validating the use of the oligonucleotide chip as a DNA fragment assay. Not only strand breaks but also enzymatic cleavage sites such as oxidized purine or pyrimidine induced by γ-ray should be measurable by DNA fragment assay using the oligonucleotide chip.

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