Safety Evaluation of Non-thermal Atmospheric Pressure Plasma Liquid Treatment

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Abstract— **We evaluate the beneficial effect of biological decontamination, and the DNA double strand breakage (DSB), for a range of relevant treatment durations. Atmospheric pressure He plasma jet, generated by a device supplied with pulsed high voltage, was employed. The treatment was applied to a mixture of buffered λDNA and *Escherichia coli* liquid solutions. After the plasma treatment, and the separation from the λDNA molecules, the cell viability (decimal reduction value, D) was obtained. Concomitantly, the length of the single λDNA molecules was measured and the DSB rate (cutting rate, k) was calculated. Lethal effect of chemicals, or other factors such as temperature, on *E. coli* has been rigorously studied. Therefore, toxicity of radicals can be compared with that of different factors by the use viability. In this study, we confirm the effectiveness of the experimental procedure using mixture of *E. coli* and λDNA in buffered solution. Using this procedure, the decimal reduction value of the viability, D, and the DNA cutting rate, k, can be obtained simultaneously. This result indicates that the toxicity of injected radicals can be quantitatively evaluated by measuring the cutting rate k, and that the evaluation can be made quickly because cultivation is not required.**

I. Introduction

Interest in applications of non-thermal plasma (NTP) in biomedicine has recently increased. Through atmospheric pressure plasma jet, subjects can be treated without thermal loading and the plasma jet length can be extended by increase in the noble gas flow. [1] For example, there is further potential to employ non-thermal plasma in chronic wound treatment, dental care, and cosmetics. [2-4]

In this context, the safety of non-thermal NTP plasma treatment and the comparison between the damage to both the bacterial cells and the DNA molecules must be addressed. It has been reported that induced damage to DNA could occur when DNA is exposed to NTP and in some cases it results in biochemical changes within the DNA. Damages include single-strand breaks (SSB) and double-strand breaks (DSB). [5] The latter is considered to be more problematic as it is harder to repair, and often leads to cell death or mutagenesis. [6-9]

Investigation of the DNA damage due to exposure to NTP is usually carried out using agarose gel electrophoresis with plasmid DNA. However, large DNA molecule analysis using this method becomes problematic. This is because the analysis requires special equipment and long run times. To overcome these difficulties, single-molecule measurement of DNA is employed. The technique was previously reported in the study of strand breaks of large DNA molecules induced by radiation [10-12] and cigarette smoke extract. [13]

Within the aforementioned considerations, there is no better way to address the NTP safety issue than using the NTP effect on *E. coli,* a strongly surviving bacteria in the human body, and well-studied already, versus the NTP effect on double strand DNA breakage. We hence provide a comparison between these two effects in the attempt to determine just how dangerous the NTP may be to the DS DNA molecule integrity. Single-molecule-based kinetic analysis of strand break on large DNA molecules induced by an NTP jet has been recently reported. [13]

Our safety evaluation uses this reported DNA analysis method as starting point. There have been no previous reports regarding the kinetics of NTP-induced DSB in DNA molecules, versus the sterilization effect.

II. MATERIALS AND METHODS

The non-thermal plasma jet treatment experiments were performed at atmospheric pressure on a mixture of 92 μl *E. coli* precipitate in 10 mM, pH 4.0 citric acid buffer and 7.75 %(v/v) of 0.8μg/μl λDNA (Nippon Gene) in TE buffer. The *E. coli* MV1184 strain had been previously introduced pGLO plasmid and incubated in LB nutrient broth. 5mg/ml arabinose had been added. The resulted concentration of living *E. coli* cells was about 5.0×10^6 cell/ml. The choice of mildly acidic medium was made to accelerate the plasma treatment. [14]

The buffered *E. coli* and λDNA mixing was performed immediately before the exposure, directly on a 0.15 mm thick microscope slide, at plasma jet marker point, as shown in Figure 1 and Figure 2.a.

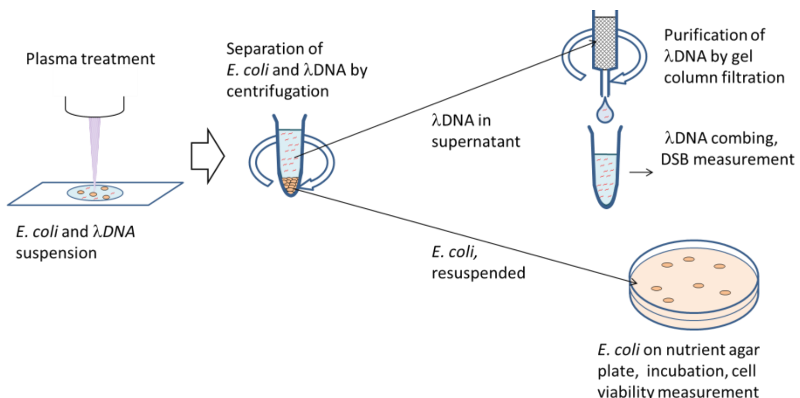

Fig. 1. Experimental protocol schematics.

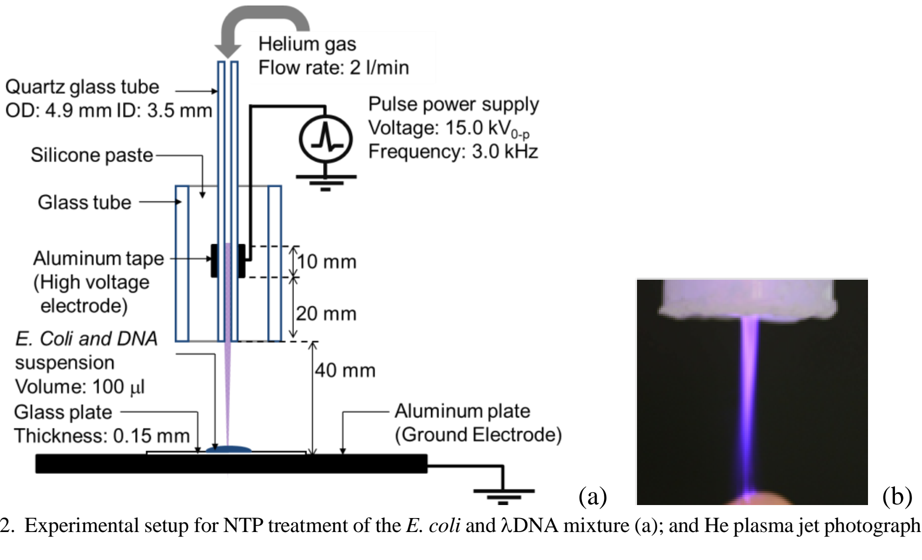

Fig. 2. Experimental setup for NTP treatment of the *E. coli* and λDNA mixture (a); and He plasma jet photograph (b).

The glass slide was positioned on an Al ground plane placed at 40 mm under the device, as illustrated in Figure 2.a.

The resulted 100 µl samples were exposed to the He plasma jet for 0 s, 30 s, 60 s, 90 s, and 120 s respectively. All experiments were performed at ambient air temperature (21.3 ˚C to 25 ˚C) and relative humidity (61.2% to 63.7%).

The schematic of the plasma jet generator and experimental set-up are shown in Figure 2.a. The plasma jet device consists of a quartz glass tube, and a 10 mm width aluminum tape wrapped at 20 mm from the tip of the glass tube. The aluminum tape electrode was connected to an adjustable high-voltage pulsed power supply (ECG-KOKUSAI, PPS-8000). Dielectric barrier discharge (DBD) was generated inside the glass tube at the applied 15 kV_{0-p}, 2.8 µs wide voltage pulses at 3.0 kHz frequency. [16] The discharge power was 8 W. The helium flow rate through the quartz tube was adjusted to 2.0 l/min before each sample exposure. These values were fixed for all experiments. Figure 2.b shows a photograph of the plasma jet generated within these parameters.

During all plasma experiments, the applied voltage and the discharge current waveform were simultaneously measured through an HV probe (Tektronix, model P6015A, 100 MΩ, 3 pF) and a current monitor (Pearson Electronics Inc., model 2877, 1 Volt/Ampere +1/-0%, 200 MHz) respectively. Both probes were connected to a digital oscilloscope (Tektronix, model DPO 2024, 200 MHz, 1 GS/s). The electric field at the exposure point was also evaluated through HV probe and oscilloscope measurement in preparation to the cell and molecule mixture exposure to plasma.

After mixing and plasma exposure, 80 µl of the sample was immediately harvested from the glass slide and, as illustrated in Figure 1, the λDNA and *E. coli* were separated through centrifugation at 2600 g, r.t. for 10 min. The λDNA in 60 µl of supernatant was immediately purified through gel column filtration equilibrated with TE buffer (CHROMA SPIN™+TE-1000 Columns, Clontech Laboratories Inc.), and stored at 4 ˚C before staining and combing.

Precipitated *E. coli* was resuspended into 60 μl distilled water and the living cell concentration was measured by standard plate count method. LB-agar Petri dishes, containing 5mg/ml arabinose and 1μg/ml ampicillin, were used for the standard plate counting.

For DS observation and antibleaching, the purified λDNA samples were stained with 10 μM YOYO-1 fluorescent dye in the presence of 10% 2-mercaptoethanol (2-ME). To 49 μl of the purified λDNA, 0.5 μl of YOYO-1 and 0.5 μl of 2-ME were added.

Single-molecule experiments were performed using molecular combing. [13] [15]. A coverslip (24 × 60 mm, 0.17 mm thickness, Matsunami) was used, without any surface treatment, for microscopic observations. With the coverslip at 45°, a 50 μl drop from the stained sample was suspended at the upper edge of the coverslip. The λDNA solution was allowed to run down the coverslip, and the fluorescently stained DNA molecules were adsorbed and combed on the glass surface. The coverslip was then dried and the sample was measured.

DNA molecules were observed under fluorescence microscope equipped with a 100 × 1.4 numerical aperture oil immersion objective lens. Fluorescent images were recorded by an EB-CCD camera connected to an AQUACOSMOS imaging system (Hamamatsu Photonics). The length of individual DNA molecules was measured using the length measurement tool in AQUACOSMOS. The contour length of more than 30 individual molecules for each combing event was measured, unless otherwise specified. The combing event was repeated three times on the surface of the same slide, thus typically more than 100 individual molecules were measured for each experimental point. This is particularly the case for the λDNA from the control sample and the λDNA exposed to plasma for 30 s.

III. RESULTS AND DISCUSSION

Before and during each plasma treatment experiment, it is important to ensure that the applied voltage amplitude, frequency and the discharge current are closely monitored, and fixed. Figure 3 shows the displayed voltage pulse and discharge current waveforms.

To calculate the power consumption, we used the oscilloscope capability to concomitantly measure the RMS voltage and the RMS current. As these values per cycle can vary, the power was calculated using the RMS values over a length of approximately 10 voltage cycles. The obtained value for the power consumption in our setup is 8 W.

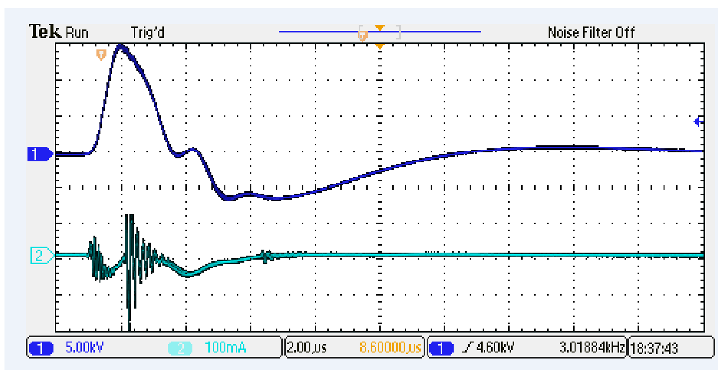

Fig. 3. Applied voltage pulse and discharge current measurement.

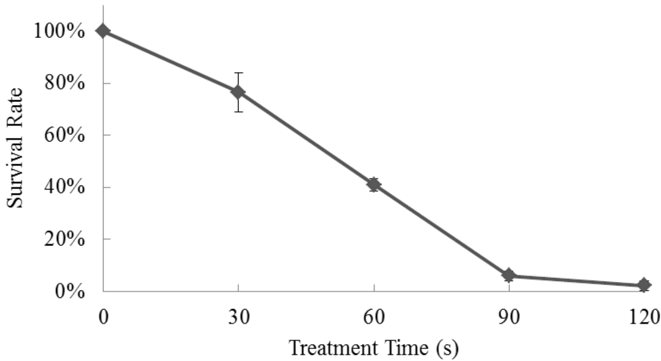

Fig. 4. *E. coli* cell viability after atmospheric pressure non-thermal plasma treatment.

For the *E coli*, the results after plasma treatment are plotted in Figure 4. The survival rate of the *E coli* was calculated by ratio to the cell concentration of the control sample. The control sample was obtained by quickly and gently mixing the buffered *E coli* and λDNA on the glass slide then immediately harvesting and separating the *E coli* cells from the λDNA molecules through the described protocol. While the He gas was on, no plasma exposure was applied to the control sample. The experiment was repeated several times.

For our experimental conditions, the *E coli* viability decreases in average by 20% after 30 s of treatment, 60% after 1 min, 94% after 90 s, and by 98% after 2 min of treatment. From both Figure 4 and the logarithmic plot of the cell viability (not shown), the decimal reduction time (D-time) is 80 s. One purpose of our work is the attempt to generalize the correlation observed between the D-value for the bacteria and the rate of DSB of the DNA molecules.

Typical results of single-molecule observations are shown in Figure 5. We observed that the number of fragmented short DNA molecules increased with exposure time. To estimate the DS breakage in the λDNA molecules, the relative length with respect to the control sample was used to estimate the change in the DNA length induced by the plasma exposure. The double-strand breakage results were then analyzed applying a mathematical model. [12-13]

$$\mathrm{L}/L_0 = 1/(n+1) \tag{1}$$

where L_0 is the DNA length before plasma exposure, L is the length after the exposure, n is the number of double-strand breakages per individual DNA molecule.

$$n = kt \tag{2}$$

where t is the exposure time and k is the rate of double-strand breakage by the plasma exposure. From Equation (1) and Equation (2),

$$k = \frac{\frac{L_0}{L} - 1}{t} \tag{3}$$

The average length of the molecules, measured for the control sample (0 s plasma exposure) and for 30 s plasma treated sample, was 21.5 μm and 19.63 μm respectively. From Equation (3), the λDNA DSB rate could be calculated. The resulted value for k is 0.19 min^{-1}.

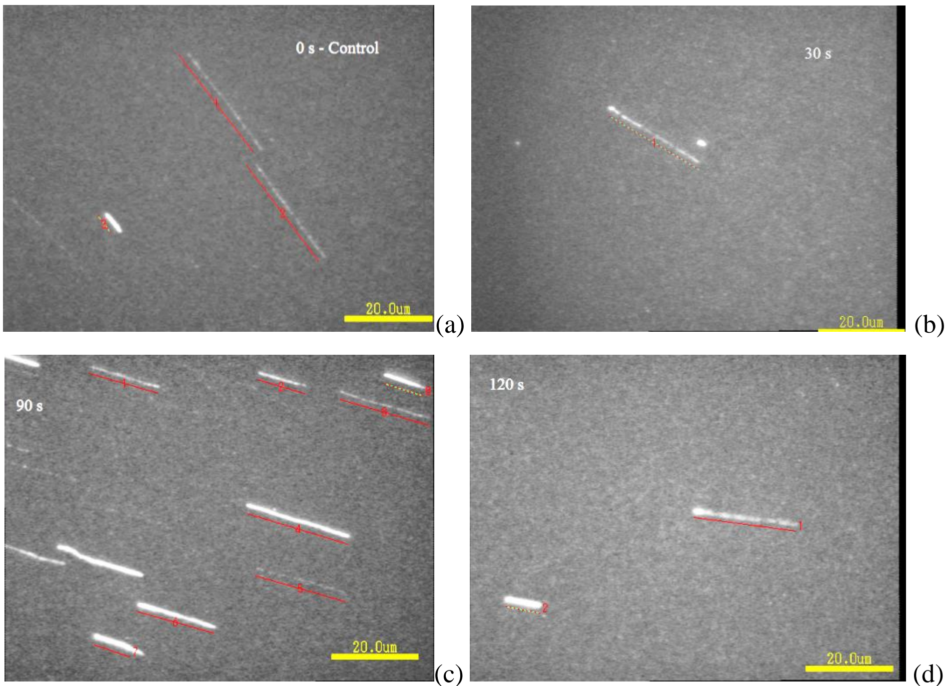

Fig. 5. Depiction of typical λDNA molecule measurement sets after plasma jet treatment and combing: (a) control sample showing long molecules, and DSB likely due to manipulation; (b) after 30 s of treatment, with apparent debris; (c) relevant molecule lengths for the calculated average after 90 s of plasma treatment, and (d) after 120 s of treatment. *Note*: spatial distribution of the stretched molecules differs on each scanned depiction, although the same concentration was used throughout for gravitational combing on the microscope slide.

The number of double-strand breaks per individual DNA molecule, n, was calculated from Equation (2) for each treatment time. The values obtained for 30 s, 60 s, 90 s and 120 s were 0.095, 0.19, 0.285 and 0.38 respectively. From our investigations thus far, there is a strong correlation between the NTP D-value for *E coli* and the number of DSB per DNA molecule. With the knowledge of the DS cutting rate, k value, for fixed experimental conditions, it could be possible to determine the plasma radical toxicity without the need to cultivate the bacteria. However, not enough data is available at this point to quantitatively report on this aspect. Experiments are underway to solidify our findings.

Figure 6 shows the relative DNA length, L/L_0, plotted as a function of the exposure time t. These results indicate that the relative DNA length decreased gradually with increasing the exposure time. The same trend in DSB was observed for the same value of k obtained after λDNA NTP jet treatment in different experimental conditions such as power, gas type and protocol. This stronger suggests the potential to model the safety evaluation for plasma liquid treatment. As seen in Figure 5, very short dot like fragments were observed, more so for the plasma treated molecules. Although the length of these small fragments could not be measured, the results show that the number of strand breaks is proportional to the exposure time.

Previous reports based on gel electrophoretic assay showed that plasma exposure to DNA could induce SSB on both the plasmid DNA and the large linear DNA.

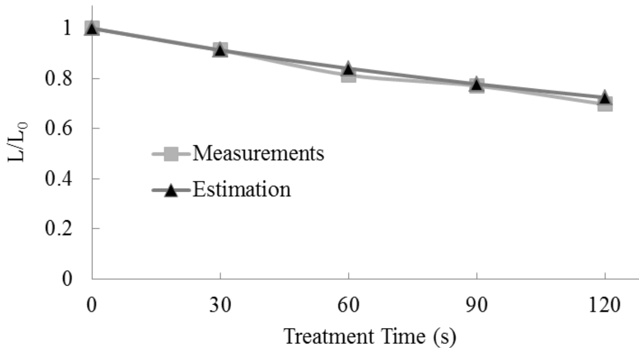

Fig. 6. Comparison between the estimated and measured rate of change of the λDNA molecule length average with the treatment duration. The estimation graph is based on the calculation of the k value, the DSB rate, at 30 s.

In our investigation, the observed shortening may include destabilization of the DNA secondary structure induced by complicated DNA damage and large linear DNA could have multiple SSB. The synergetic effect of these SSB and other factors, such as mechanical stress due to Brownian motion, experimental manipulation, and the molecular combing, could cause fragmentation of the large linear DNA. This suggests that the observed rate of double-strand breakage induced by the NTP jet is the overall rate of these factors. However, as Figure 5 suggests, some of these effects are also present in the control.

The damaging effect of UV on DNA is known as in the range 200 nm to 280 nm. However, the UV radiation level was too low to be detected. Several research groups reported that strand breakages might be oxidative damage induced by reactive oxygen species. [4-9] In some cases, more than 60% of the damage during plasma irradiation is related to the interaction of excited or/and reactive species. [7] Additionally, a helium plasma jet comprises of helium ions and electrons, with the electrons accelerated on water molecules in the aqueous solution to generate OH. Hence, OH radicals are considered to contribute to the strand breakages in this experiment (although measurements of the concentrations of reactive species and the reaction pathways have not been performed). In addition, the relationship between the obtained rate of double-strand breakage and DNA damage in cells remains unknown.

IV. CONCLUSION

The safety of NTP treatment on liquids can conveniently be evaluated by comparing the already rigorously verified *E. coli* model with the DSB effect on λDNA length.

(1) The safety of NTP jet treatment on liquids in relation to the beneficial effect of *E coli* sterilization is for the first time evaluated. While the *E coli* viability dramatically decreases after a certain treatment time, the number of double strand breaks (DSB) induced by the plasma treatment is proportional to the exposure time by the cutting rate k.

(2) A valid estimation of the λDNA DSB rate per min, k, is obtained and used in the safety evaluation for all treatment times in fixed conditions. This rate could be used to determine the *E. coli* D-value, case in which the bacteria cultivation would no longer be necessary.

(3) Further investigation, currently underway, is necessary to quantitatively correlate the viability of *E. coli* and the DNA double-strand fragmentation as result of non-thermal atmospheric pressure plasma jet treatment of contaminated liquids.

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