Analysis of the inactivation mechanism of bacteriophage by Atmospheric barrier discharge

Takuya Miura, Hachiro Yasuda, Hirofumi Kurita, Kazunori Takashima and Akira Mizuno

***Abstract*— The inactivation mechanism of virus and bacteria by atmospheric discharge plasma has been studied actively. However, predominant factors in the inactivation are not clear at all. Because the atmospheric discharge plasma includes a lot of possible inactivation factors such as active oxygen species, ozone and ultraviolet rays, it is difficult to analyze each effect separately.**

In this study, bacteriophage lambda was used as the simplest model biological samples to analyze the inactivation mechanism by atmospheric discharge plasma. When bacteriophage having only DNA and coat proteins is inactivated by DBD, damage should exist on DNA or coat proteins. In the experiment using bacteriophage, it is able to extract DNA from the bacteriophage so that the damaged coat proteins can be removed to avoid the effect of the proteins. And, DNA which is extracted from plasma-inactivated phage can be assayed its plaque forming activity. Using this assay, the damages on DNA and coat proteins have been determined separately. The results show that inactivation of lambda phage by atmospheric DBD was attributed to the damage mainly of coat protein. The damage of lambda DNA was negligible in the early stage of the inactivation.

***Key word*—Non-thermal plasma, Plasma sterilization, Virus, Bacteriophage**

I. INTRODUCTION

In the fields of food processing and medical treatment, sterilization is one of the most important process for guaranteeing safety service. Autoclaving, ethylene oxide gas, hydrogen peroxide and radioactive ray are used as conventional sterilization methods. However, these methods have problems such as taking long treatment time and remaining of poisonous matter. Currently, application of electric discharge plasma for sterilization is studied widely.

Non-thermal atmospheric pressure plasmas have recently been applied in the biomedical field. The cold atmospheric plasma provide a rich environment of charged particles, reactive ground-state species, excited species and metastables, UV photons, and intense electric fields, any of which can in principle significantly affect bacterial and mammalian cells. Several types of devices for low temperature atmospheric plasma have been developed and applied to medical research such as sterilization [1]-[7], wound healing [8]-[11], tissue engineering [12],[13], DNA transfection [14] and induction of apoptosis [15]. Dielectric Barrier Discharge (DBD) is one of the devices for generating stable plasma at atmospheric pressure [1],[16],[17].

Most studied and comprehensively established is the outstanding capability of cold plasmas to inactivate microorganisms [18]. The distinctive features of plasma sterilization which do not use bactericides and can be performed at low gas temperature seem to be applied to sterilization of surgical instruments and medical devices, decontamination of foods, inactivation of bacteria on living tissues and wounds, and decontamination of room air [19]. While the field of plasma decontamination is expanding, to understand the mechanisms of plasma inactivation are yet to be conclusively established. It is estimated that the inactivation of bacteria by atmospheric discharge plasma causes cell wall destruction by potential difference and cell denaturalization by electric current. However, the factors essential for inactivation were not completely known yet. Because the atmospheric discharge plasma contains a lot of inactivation factors such as active oxygen species, ozone and ultraviolet rays, it is difficult to analyze single effect of these. Moreover, the fact that excessive plasma exposure disintegrates components of virus and bacteria, also make it difficult to analyze the mechanism of inactivation.

For carrying forward the plasma applications to medical field, it is very important to know the effects caused by plasma on the cells and cellular components, especially membranes, proteins and DNA at the molecular level [20]. But the complexity of both the donor plasma source and the recipient cell components has so far made it difficult to analyze biological mechanism of plasma-inactivation.

We have applied atmospheric DBD to the wet state of *Escherichia coli* cells. Upon DBD treatment, *E. coli* was immediately inactivated and the states of different biological components were monitored during the course of inactivation. Only minor and slow degradation of proteins, DNA, and membranes was observed in the early stage, and a remarkable degradation was seen after the completion of sterilization. Analysis of GFP recombinantly introduced into *E. coli* cells proved that the DBD has a prominent protein denaturation activity without affecting peptide bonds. The irreversible denaturation of proteins, seen in the early stage of DBD

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application, seemed to play a central role in inactivation of bacteria [20].

Using bacteriophages to investigate plasma-inactivation, we have developed a method which enables to estimate the damage of DNA and protein separately. Most of bacteriophages are composed of only coat proteins and DNA. In the infection process, coat proteins are cast off outside the cell and DNA intrudes inside [21]. It is also possible to transfect naked phage DNA into bacterial cells for phage propagation [22]. Therefore, DNA extracted from plasma-inactivated phages can be examined its plaque forming activity by DNA transfection. This assay is influenced by only damage in DNA and damage in proteins is excluded completely. We applied this technique to lambda phage-*E. coli*. systems. From the plasma-treated lambda phage, DNA was extracted and subjected to *in vitro* packaging reactions [23] [24]. These re-packaged phages recovered their plaque forming ability to great extent. The results show that inactivation of lambda phage by atmospheric DBD was attributed to the damage of coat proteins. Though DNA damage gradually accumulates with increasing plasma treatment time, most of them were introduced after the preceding inactivation caused by protein damage.

II. EXPERIMENTAL PART

A. *Application of atmospheric DBD plasma*

Figure 1 shows the experimental system of atmospheric DBD. Figure 2 illustrates the DBD reactor electrodes used in this study. Stainless steel mesh (ϕ 50 mm, 20 mesh) and an aluminum plate were used as high-voltage and ground electrodes, respectively. A teflon sheet (2mm-thick) was set on the high voltage electrode as a dielectric barrier. A high voltage AC power supply (Kasuga-denki AGF-010) was used to generate the uniform filamentous streamers in the atmospheric air gap (3mm). In this report, discharge experiments were performed under fixed electrical conditions except during operating time. The peak-to-peak voltage, the frequency of the applied voltage, and the input power were 40kV$_{p\text{-}p}$, 2kHz, and 8.7W, respectively. The applied voltage was adjusted using an oscilloscope. The power consumption was calculated using the Lissajous figure method from simple harmonic oscillations.

A piece of PET film (30×65mm, 0.1 mm thickness) was soaked in 0.1% gelatin for 5 min and air dried for 24 h under UV light. A 20 μ L aliquot of sample solution was spotted and widely spread to 3–4 cm^2 on the PET film and the atmospheric plasma was immediately applied for the intended time. After the DBD application, the sample solution was recovered into a microtube with additional washing with 40 μ L of distilled water on the surface of the PET film. The recovered samples were used directly for enumeration of survivals or assay of DNA damage after extraction of the phage DNA.

B. *Biological materials*

In this study, bacteriophage lambda (λ phage) was used. Bacteriophage is a kind of viruses which specifically infect to

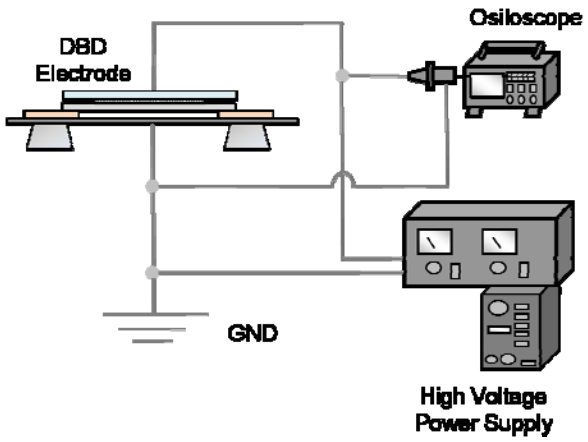

Fig. 1 Experimental system for DBD application

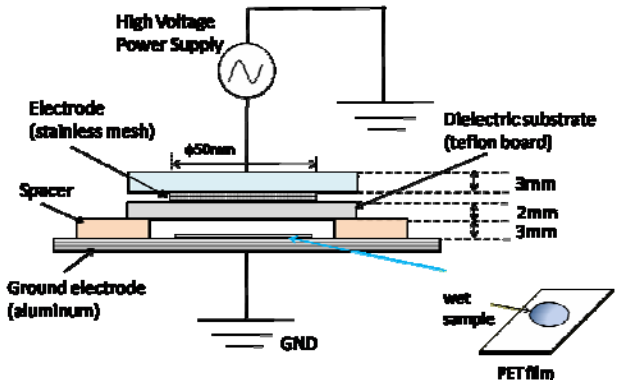

Fig. 2 Atmospheric DBD reactor

bacteria. λ phage (λ CI857Sam7) was induced from λ -lysogen cells, *E.coli* M65 (λ). The λ lysogen cells were inoculated to 200ml of LB medium in a 1 litter flask and shaken at 32℃. When OD650 reached 0.5, the cultivation temperature was quickly shifted to 42℃ and shaken for 20min and then shaken for 3hrs at 40 ℃. The cells were harvested by centrifugation at 8000×g for 5min and re-suspended with 10ml of SM buffer (20mM Tris·HCl (pH 7.5), 100mM NaCl, 10mM MgSO4, 0.01% gelatin). The cells were lysed by adding 0.1ml of chloroform, 10 μ l of 2mg/ml of pancreatic DNase and gentle shaking at 37℃ for 20mim. The cell lysate was centrifuged at 10000 × g for 10min and recovered the supernatant. Further purification of the λ phage in the lysate by stepwise CsCl density gradient centrifugation and CsCl equilibrium density gradient centrifugation has been done according to the literature protocol [25], except that phage was finally dialyzed against 100mM Tris · HCl (pH8.0), 1mM MgCl2. Before application of DBD, the phage sample (3× 10^{12}/mL) was diluted with 100mM Tris-HCl (pH8.0), 1mM of MgSO^4 and adjusted the viable phage concentration to 2× 10^9/mL

In vitro DNA packaging extracts (Giga Pack Gold Ⅲ) were purchased from Stratagene Co.

C. *Titration of λ phage*

Titration of the DBD treated λ phage was done as follows: As indicator cells, *E.coli* Y-mel (supF) was cultivated in LB medium at 32℃ over night. 0.1ml of the serially diluted λ phage solution was mixed with 0.1ml of the indicator cell suspension and kept at 37℃ for 10mim. 3ml of LB soft agar kept at 45℃ was added to the mixture of infection and poured on a LB plate. Phage plaques on the plates were counted after incubation at 40℃ for 40hrs.

All of the survival measurement experiments were duplicated independently and the mean value was adopted.

D. *Assay of DNA damage*

In this study, *in vitro* DNA packaging was used as an analytic tool. *In vitro* DNA packaging is a method to form infectious λ phage particle by mixing purified DNA with *in vitro* DNA packaging extracts which contain λ coat proteins.

From 40 μL each of DBD treated samples, phage DNA was obtained by serial extraction with phenol, phenol-chloroform, and chloroform as described in the book [24]. After precipitation with ethanol and drying up, the DNA was dissolved in 30 μL of 10mM Tris･HCl(pH8.0), 1mM EDTA. 1 μL of the DNA extracted from DBD-treated λ phage was mixed with 8 μL of the packaging extracts, and the mixture was incubated at 22℃ for 2 hours. To the each mixture, 200 μL of SM buffer (20mM Tris･HCl (pH 7.5), 100mM NaCl, 10mM MgSO4, 0.01% gelatin) and 8 μL of chloroform was added and centrifuged at 8000×g for 2min. The supernatant containing the newly packaged phage was rendered for titration.

E. *Electrophoresis*

Analysis of proteins of λ phage by SDS polyacrylamide gel electrophoresis was performed according to the manuscript [26].

The DNA of DBD treated λ phage was extracted by adding SDS and EDTA to the final concentration of 0.8% and 10mM respectively, and heating at 70℃ for 10mim. The DNA was fractionated by 0.3% agarose gel using 1×TBE (89mM Tris-borate buffer (pH 8.3), 2mM EDTA). DNA samples of λ phage were incubated at 65℃ for 5min just prior to application of the agarose gel. Electrophoresis was subjected for 2hour at 4℃ with applying DC 50V. Staining the gel with ethidium bromide and taking a photograph with Polaroid camera under UV irradiation were done as described [24].

III. RESULTS AND DISCUSSIONS

A. *Plasma inactivation of lambda phage*

In the application of atmospheric DBD for bacteriophage inactivation, we chose wet state of samples because biologically active state of the phages can be investigated. Wet samples have also advantages that surrounding conditions of the cells can be controlled by adding chemicals to the solution.

To obtain the reproducible experimental results of inactivation in wet samples, it was necessary to spread the sample solution on the PET film widely and keep the thickness of the water layer less than 0.1mm. The samples did not evaporate significantly during the DBD application, which suggested the phages were kept at a low and harmless temperature. All the samples for DBD application contained 100mM Tris･HCl (pH8.0) to avoid a rapid dropping down from the initial neutral pH. The phenomena of increasing acidity of the samples may be mainly caused by NOx gas produced in the air field of the discharge [4],[27]. NOx will form nitric acid or nitrous acid after dissolving in water. In our buffering condition, in 100mM Tris･HCl (pH8.0), decrease of pH value was delayed and neutral pH of the samples was guaranteed until 20 sec discharge, but 30 sec discharge led the sample solution to acidic around pH 4.5 (Figure 3). Therefor, we relied on the experimental data of up to 20sec discharge which are free from the secondary effect of air plasma.

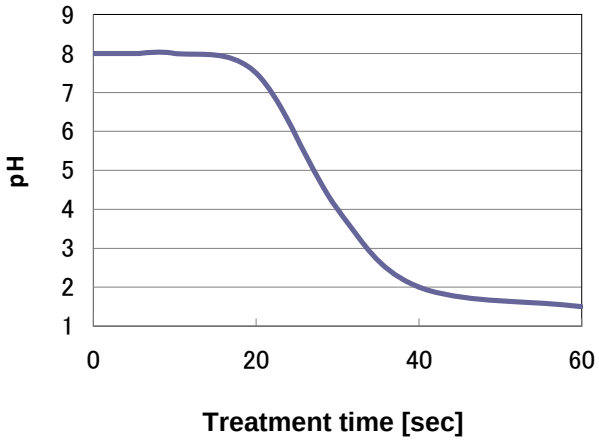

Fig. 3 pH of samples under DBD treatment

Damage to the cell membrane have been thought to be essential for bacterial inactivation by low temperature plasma [28],[29]. But bacteriophages usually do not have a membrane and consist of only proteins and nucleic acids. It is interesting to investigate the effect of plasma on the membrane-free bacteriophages [1],[30].

Figure 4 shows the inactivation profile (survival curves) of the λ phage after time-lapse treatment with the DBD. The number of infectious phages decreased quickly and sterilization (6-orders of magnitude inactiovation) was achieved by 20sec discharge treatment. The profile exhibited the characteristic of a single slope curve [3],[4],[31] and the D-value was about 3sec. Damage which brought this inactivation must exist in coat proteins or phage DNA or both of them. Therefore, the integrity of proteins or DNA of plasma treated λ phages was investigated by electrophoresis.

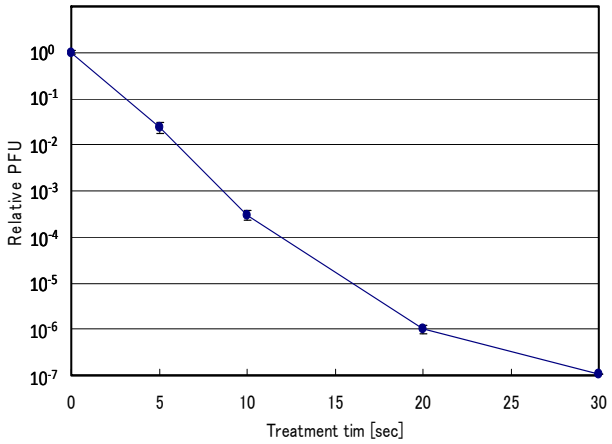

Fig. 4 Inactivation profile of DBD treated λ phage
PFU : plaque forming unit

Figure 5 shows the analysis of proteins from the DBD treated λ phages by SDS polyacrylamide gel electrophoresis. λ phage has about 20 genes of coat proteins [32], but only two major proteins were detectable in the DBD treated samples. These coat proteins did not degrade prominently until 20sec of plasma treatment. Compared to their rapid inactivation, considerable amount of the full length coat proteins remained even 20sec application of the plasma which brought 6-log reduction of active phages. So, fragmentation of the coat proteins does not seem to be primary cause of inactivation by cold plasma.

Figure 6 shows the analysis of DNA from the DBD treated λ phages by 0.3% agarose gel electrophoresis. The phage DNA, similar to the coat proteins, did not degrade prominently compared to the rapid inactivation. Considerable amount of the full length phage DNA remained even DBD treatment for 20sec which brought 6-log reduction of active phages. Therefore, fragmentation of the phage DNA also may not be responsible for the inactivation by cold plasma. Degradation was seen in the 30sec treated sample which seems to be caused by increased acidity of the sample because DNA is labile to acid.

These analysis by electrophoresis could not specify the damage caused the inactivation. But the results from electrophoresis suggested that molecular damage responsible for the inactivation was not degradation of proteins or DNA but denaturation or chemical modification of them. The damage may possibly be brought by reactive oxygen species produced in the non-thermal atmospheric plasma.

B. Evaluation of DNA damage

Figure 7 illustrates the method to estimate the DNA-specific damage in plasma applied λ phages. Putative damage is introduced in both protein and DNA of the plasma treated phages (Phage A). DNA is extracted from Phage A and packaged *in vitro* to form newly packaged phages (Phage B). Phage B does not have protein damage and carries only DNA damage originated from Phage A. Therefore, all of the inactivation factors in Phage B originate DNA damage brought

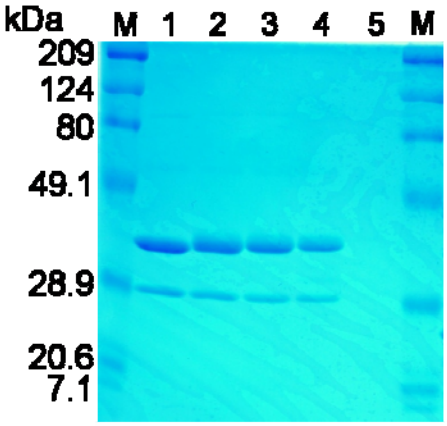

Fig. 5 SDS-polyacrylamide gel electrophoresis of discharged λ phage.
Proteins were extracted and separated in 14% gel before staining with Coomassie Brilliant Blue (CBB). Lane M is a protein standards marker. Lanes 1-5 represent the coat proteins from the phages treated with DBD for 0, 5, 10, 20 and 30 sec, respectively.

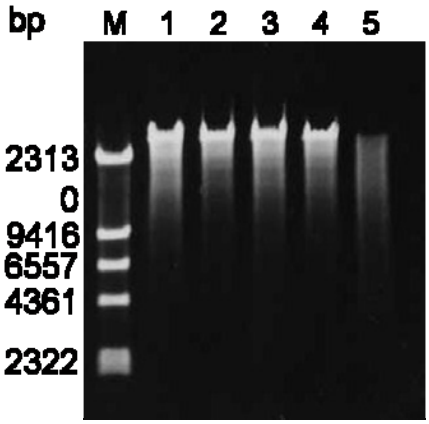

Fig. 6 Agarose gel electrophoresis of DNA from discharged λ phage
DNA was separated in 0.3% agarose gel. Lane M is HindⅢ digests of λ DNA Lanes 1-5 represent the λ DNA from the phage discharged by atmospheric DBD for 0, 5, 10, 20 and 30 sec, respectively.

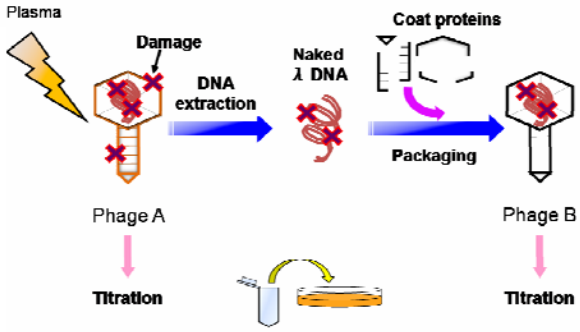

Fig. 7 Estimation of the DNA-specific damage in plasma treated λ phages.

from Phage A. Because re-packaging procedure of λ phage usually decreases the efficiency of infection, absolute number of active phages (phage titer) in Phage A and Phage B can not be compared directly. Comparison of the normalized survival curves from Phage A and Phage B enables to evaluate the DNA damage and the protein damage.

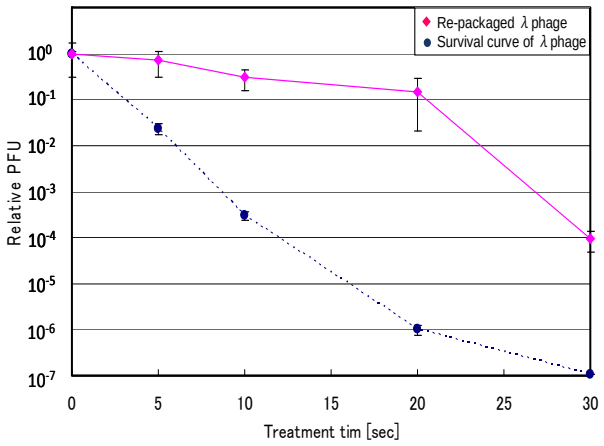

Fig. 8 Survival curves of the re-packaged λ phage. Dotted line represents the same line shown in Fig.4.

Figure 8 shows the actual survival curves obtained from the re-packaged λ phages along with the survival curves of plasma treated phages. These survival curves were normalized with each control sample (0sec samples) to give the PFU (plaque forming unit) value of 10^0. The profile exhibited the characteristic of a single slope curve until 20sec discharge and the D value was about 25sec. Large D value of the re-packaged phages means very slow decrease of infective phages until 20sec. The results indicate that the DNA damage introduced by plasma was very small and did not accumulate prominently with increase of the discharge time. It seems that all of the initial damage for inactivation in phages introduced by plasma was protein damage, and DNA damage was marked on the phages already inactivated by protein damage. So, it is concluded that inactivation of λ phage by atmospheric DBD was attributed to the damage of coat proteins. The damage of λ DNA was negligible in the early stage of the inactivation.

The damage responsible for phage inactivation can be recognized only when the assay of phage viability was carried out. Therefore, the damage for inactivation may not be directly correlated to the amount of the molecular damage. For example, molecular damage introduced in the binding protein which interacts with phage receptors on *E. coli* cell surface may largely affect to the inactivation. Moreover, it should be counted that cells have a highly developed DNA repair system. Molecular damage of DNA might have been repaired effectively than that of proteins.

Anyway, it will be the first time that showed the evidences of primary damage which affected to plasma inactivation. The bio-assay of DNA damage has enough reproducibility when the strains of phage and host cell were fixed. And this assay can be applied to not only plasma inactivation but also any type of inactivation step of bacteriophages. Application of the DNA damage assay to other plasma sources or different sets of bacteriophages and host cells may bring valuable insights into the mechanism of plasma inactivation.

IV. CONCLUSION

Using bacterial virus (bacteriophage lambda), we have developed an assay method which enables to evaluate the DNA specific damage in the process of inactivation. The method contains extraction of DNA from inactivated bacteriophage and transfection of the DNA to the host bacteria. This assay was applied to the bacteriophage (λ phage) which was inactivated by the treatment of non-thermal atmospheric air plasma. It was found that inactivation of λ phage by atmospheric DBD was attributed to the damage of coat proteins. The damage of λ DNA was negligible in the early stage of the inactivation. It seemed to be the first experimental demonstration that specified the primary damage which brought the plasma inactivation of microorganisms.

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