Plasmid DNA Damage Following Exposure to Atmospheric Pressure Nonthermal Plasma: Kinetics and Influence of Oxygen Admixture

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ABSTRACT: The nature and kinetics of plasmid DNA damage after DNA exposure to a kHz-driven atmospheric pressure nonthermal plasma jet has been investigated. Both single-strand break (SSB) and double-strand break (DSB) processes are reported here. While SSB had a higher rate constant, DSB is recognized to be more significant in living systems, often resulting in loss of viability. In a helium-operated plasma jet, adding oxygen to the feed gas resulted in higher rates of DNA DSB, which increased linearly with increasing oxygen content, up to an optimum level of 0.75% oxygen, after which the DSB rate decreased slightly, indicating an essential role for reactive oxygen species in the rapid degradation of DNA.

KEY WORDS: Plasmid, DNA, DNA damage, nonthermal plasma, atmospheric pressure plasma, plasma medicine, plasma sterilisation, ozone, singlet delta oxygen.

I. INTRODUCTION

Plasma medicine has been an active research area since the beginning of this millennium, with a special emphasis on atmospheric pressure nonthermal plasma (APNTP) or, as it is also known, cold atmospheric plasma. Thus far, this kind of plasma has been investigated for use in a wide range of medical applications including cancer treatment, blood coagulation, wound healing, surface decontamination, and sterilization.1–4

APNTP’s excited, dissociated, and ionized gas molecules produce a mixture of chemically reactive species.5 A common practice is to use a noble gas (i.e., helium or argon) to create the plasma and to add a small fraction of a molecular gas (usually oxygen or nitrogen) to “enrich” the chemistry of this plasma; a wide range of reactive oxygen and nitrogen species can be generated in this way. Atomic oxygen, singlet delta oxygen (SDO), ozone, hydroxyl radicals, and other reactive oxygen species have
been detected in the effluent of cold plasma jets operating in helium with a small fraction of oxygen. The nature and densities of these reactive species can be “tuned” by adjusting different internal and external parameters of the plasma. One of the most important parameters, which greatly affects plasma chemistry, is the gas mixture used to produce the plasma. Because reactive oxygen and nitrogen species produced in the plasma are believed to mediate at least a major part of its biological impact, it is essential to evaluate important changes in biological responses to plasma exposure caused by varying the plasma gas mixture and to correlate these changes with the diagnostics of plasma chemistry.

As with any other medical treatment, safety and efficacy are the crucial issues that need to be addressed before any successful translation of plasma treatment from the laboratory to clinical applications. Deoxyribonucleic acid (DNA) is a vital component of living cells, and DNA damage can result in the death of cells or in significant deviation from normal function. These detrimental effects, which should ideally be minimized in host cells, may be desirable in the case of cells targeted for destruction by plasma treatment, such as microbes and malignant cells, where DNA damage is believed to be an important part of the cell destruction mechanism. As implied above, the damaging effects exerted by different APNTP systems on DNA are largely associated with both safety and efficacy issues in plasma medicine, which makes the understanding of these effects an essential requirement for any successful plasma application in this arena.

Despite the importance of APNTP-mediated DNA damage, few studies have been dedicated to the detailed investigation of this issue. A study using a kHz-driven APNTP jet operated at 8 kHz with 2 SLM helium and 1% O₂ admixture has been reported to cause conformational changes to plasmid DNA exposed in liquid samples, after plasma exposure. Although this report contained only a semi-quantitative evaluation, Yan et al. showed comparable DNA damage at an exposure time scale similar to that of our study. Dry DNA samples exposed to a helium-only APNTP jet, operated at 3.2 kHz, showed a similar trend of damage but at a slower rate. In those studies, no direct quantitative correlation was made between DNA damage and any of the reactive species produced in the plasma. However, O’Connell et al. reported a good correlation between the rate of DSB occurring to plasmid DNA in aqueous samples after plasma exposure and atomic oxygen densities produced in a radiofrequency (RF)-driven (13.56 MHz) plasma jet operated with a 1 SLM helium and 0.5% oxygen admixture. Nevertheless, atomic oxygen density was adjusted by varying the RF power input rather than changing the amount of added oxygen. O’Connell et al. reported that varying rates of DNA strand breakage were dependent on the buffer used to prepare the DNA samples. The overall trend was in good agreement with our results. In this study, we examined the influence of varying the concentration of oxygen in the feed gas, one of the critical parameters in APNTP production, on the kinetics of plasma-mediated DNA damage.

The objective of this study was to investigate the nature and kinetics of plasmid DNA damage mediated by a kHz-driven atmospheric pressure nonthermal plasma jet.
Particular emphasis was placed on the influence of the concentration of oxygen in the helium feed gas. The ultimate aim of this study is to contribute to establishing correlation between different plasma parameters (in this case operating gas mixture) with their potential biological effects (e.g., DNA damage), which may prove to be a useful cell-free surrogate of biological toxicity for rapid optimization of plasma sources. This approach should facilitate the development of a predictive indicator that can be used to tailor APNTP systems for different biomedical applications in a more controlled manner.

II. MATERIALS AND METHODS

A. Plasma Source

The plasma source used in this study was based on the source previously reported by Teschke et al.\textsuperscript{18} and described elsewhere.\textsuperscript{3,4,13,19–21} Briefly, this plasma jet consists of a quartz tube with inner and outer diameters of 4 mm and 6 mm, respectively. Two copper electrodes (2 mm wide) encircle the tube and are spaced 25 mm apart. The upstream electrode is grounded and the downstream electrode, located 10 mm from the end of the quartz tube, is connected to a pulsed voltage source (Haiden PHK-2k) that delivers a 6-kV pulse at a repetition frequency of 20 kHz, with rise to fall time of 1.8 μs. Here, the plasma jet was operated with a helium flow rate of 2 standard liters per minute (SLM) and with oxygen admixtures varying from 0 to 1.0%.

B. Treatment Conditions and Gel Electrophoresis Analysis

Plasmid DNA (pBR322 DNA plasmid vector, Fermentas), which was originally isolated from \textit{Escherichia coli} and is 4361 bp (base pairs) in length, was used in this study. Working plasmid solution (20 ng/μl) was prepared in phosphate-buffered saline (PBS), then aliquots of 20 μl of this working solution (which contains 400 ng of plasmid DNA in total) were exposed to the plasma plume for 0, 10, 20, 30, 40, 50 and 60 seconds at a distance of 10 mm between the sample and the end of plasma tube. After plasma exposure, DNA was analyzed by gel electrophoresis using 1% agarose gel with 0.5 μg/ml ethidium bromide in 1X TAE (Tris-Acetate-EDTA) buffer. Images of each gel, showing distinct fluorescent bands for different plasmid conformations, were taken using Benchtop UV Transilluminator (BioDoc-It™ Imaging System, UVP, Cambridge, UK) and were used in further quantitative analysis. The relative abundance of each plasmid form present after each exposure time was calculated by quantitative band analysis using Image J software.\textsuperscript{22} Rate constants for both single-strand break (SSB) and double-strand break (DSB) processes were calculated by fitting the obtained results to the model equations reported by McMahon and Currell (2011).\textsuperscript{23} Although these model equations were developed to evaluate the DNA damage caused by ionizing radiation, they were report-
ed to correlate well with the kinetics of DNA damage caused by atmospheric pressure plasma.\(^{16}\)

III. RESULTS AND DISCUSSION

Bacterial plasmids are small, circular, extrachromosomal, double-stranded DNA molecules that usually carry genes for antibiotic resistance and other complementary functions.\(^{24}\) Normally, intact plasmids occur mainly in a supercoiled (SC) conformation, with an SSB on either strand of the double-stranded plasmid, resulting in the release of torsional energy stored in the SC form. Subsequent relaxation of the SC plasmid results in the conversion of this relatively compact conformation into an open circular (OC) form. If a DSB occurs or if another SSB event occurs on the opposite strand at or near a previous SSB incident, the plasmid will be converted to a linear (LIN) DNA form.\(^{15}\) Each of these conformations shows a distinct band when analyzed by gel electrophore-

![Gel electrophoresis images](image)

**FIG. 1:** Gel electrophoresis images of plasmid DNA exposed to plasma operating with (A) 0% and (B) 0.75% oxygen admixtures. Plasmid DNA can exhibit three different structural conformations each with a distinct band as follows: supercoiled (SC); lower band, open circular (OC); upper band, and relaxed linear (LIN); middle band
sis, which facilitates the quantification of the relative amounts of the different plasmid forms in a sensitive and precise manner.

Figure 1 shows the three distinct bands for the plasma-exposed plasmid DNA observed on the gel; the fastest, the intermediate, and the slowest migrating bands correspond to supercoiled, linear, and open circular plasmid conformations, respectively. As shown in Figure 1, plasmid exposure to APNTP resulted in rapid and significant DNA damage. Figure 2 shows that the intact SC form was clearly declining as exposure time increased, and, because degradation is the only potential fate for SC form, appearance of the OC conformation exhibited a more complicated trend, with an initial increase followed by a drop in its relative amount. The trend for the appearance of the OC con-

FIG. 2: Relative form abundance of plasmid DNA exposed to plasma operating with 0% and 0.75% oxygen admixtures. Individual points represent the average of three experimental values. Solid (for 0.75% O₂) and dashed (for 0% O₂) lines are the best fit according to model equations reported by McMahon & Currell. Error bars represent SEM

<table>
<thead>
<tr>
<th>Percentage Oxygen</th>
<th>SSB</th>
<th>DSB</th>
<th>DSB/SSB</th>
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<tbody>
<tr>
<td>0.00</td>
<td>0.059±0.004</td>
<td>0.0038±0.0004</td>
<td>0.064</td>
</tr>
<tr>
<td>0.25</td>
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<td>0.0049±0.0002</td>
<td>0.072</td>
</tr>
<tr>
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<td>0.0075±0.0008</td>
<td>0.093</td>
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<tr>
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<td>0.125</td>
</tr>
<tr>
<td>1.00</td>
<td>0.071±0.004</td>
<td>0.0087±0.0006</td>
<td>0.123</td>
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formation can be explained by the fact that at any instance OC can be produced from the SC form by an SSB incident and can also be degraded into LIN form by either SSB or DSB (but mainly DSB). At early exposure times where SC amount is high and because the rate of SSB is approximately an order of magnitude higher than that of DSB (although both are assumed to be first-order processes), the production rate of OC exceeds its turnover rate, causing its amount to increase (Table 1). Once OC abundance exceeds that of SC far enough to compensate for the difference between SSB and DSB rates, its degradation will be predominant, with the resulting decline in its overall amount. LIN fraction increased continuously with plasma exposure because it is irreversibly produced from both SC and OC forms. Previous studies have reported similar damage behavior, albeit with different time scales, after exposure of plasmid DNA to three other atmospheric pressure plasma jets.\textsuperscript{14–17}

The previous discussion assumes that plasmid DNA can only exhibit the three forms mentioned therein; however, this is not representative of the whole scenario. Figure 1 also reveals the presence of a ‘DNA smear,’ especially at longer exposure times. This smear represents the fraction of plasmid DNA that has been successively damaged and fragmented into smaller polynucleotide fractions with a broad range of lengths. The highly variable length of fractions prevents the appearance of a discrete band corresponding to the fraction of plasmid on the electrophoresis gel, which makes it difficult, if not impossible, to accurately quantify using the method adopted in this study. For simplicity of analysis and because plasmid fragmentation results primarily from multiple SSBs and DSBs for which estimation is possible based on the relative abundance of the other three plasmid forms, the fragmented proportion of the plasmid is disregarded in the remainder of this study.

Figure 3 illustrates how the percentage oxygen, added to the helium operating gas, influences the rate of DSB process. Although there was obvious damage with the plas-
ma operating only in helium gas, the rate of DSB increased linearly with the oxygen content, reaching more than double at 0.75%. After 0.75%, further oxygen increase did not enhance DSB rate but actually caused a slight reduction in it. The enhancement in the SSB rate was not as significant as it was in the DSB rate, but the DSB to SSB ratio (Table 1) did increase in the same manner as the DSB rate. Although both breakage processes (SSB and DSB) can have negative consequences on the structural integrity and functionality of DNA molecules (i.e., SSBs were higher than DSBs by approximately one order of magnitude after plasma exposure), in a cellular environment, SSBs are usually not lethal because they are reversible and easily fixed by cellular repair enzymes.\textsuperscript{25,26} On the other hand, DSBs are more difficult for the cell to effectively repair, especially when they occur on vital DNA molecules (chromosomal DNA) and may therefore prove lethal to viable cells\textsuperscript{27–29}; therefore, plasma produced with higher oxygen content may exert enhanced lethality toward viable cells.

Because plasmas are characterized by a complex chemical environment, it is unlikely that the observed DNA damage is mediated solely by one reactive species; rather, it is anticipated that a synergistic effect between multiple plasma constituents exists. Therefore, it is necessary to investigate the potential effects and interactions of more plasma-generated species to gain a more comprehensive understanding of this complex phenomenon and the ability to control it.

IV. CONCLUSIONS

In this study, a kHz-driven, atmospheric-pressure, nonthermal plasma jet showed a significant damaging effect on bacterial plasmid DNA, resulting in both SSBs and DSBs in this macromolecule. Although we investigated the plasma-mediated damage occurring on plasmid DNA in this study, it is likely that the nature and kinetics of the processes leading to DNA damage will be analogous to damage occurring to chromosomal DNA or other DNA molecules; they all share the same basic chemical structure and undergo similar interactions with oxidative species. While not all DNA molecules have SC, OC, and/or LIN conformations, all double-stranded DNA can potentially suffer from SSBs and DSBs. Although the trend and optimum percentage oxygen presented in this study may vary among different plasma systems, the results of this study demonstrate that the amount of added oxygen directly affects DNA damage. Because the integrity of DNA is of critical importance for any living cell, investigating potential DNA damage, particularly in regard to DSB, resulting from any plasma system intended for use in medical applications is essential. The parameters of each plasma system must be optimized, including the content of oxygen or other molecular gases, to achieve the desired DNA effect and to minimize collateral damage in nontargeted cells. Addressing individual and synergistic involvement of different plasma-produced species, especially reactive oxygen and nitrogen species, and linking their densities to different plasma parameters can also provide a platform for predicting the potential for DNA damage caused by a certain plasma system. Such an approach may allow a quick, empirical predictive method to
adjust the different parameters of a plasma system, tailoring it to suit specific biomedical applications.

REFERENCES

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