In vitro characterization of bacterial macromolecule damage induced by low-pressure VHF-CCP plasma

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Abstract: **Low-pressure plasmas have great potential for bacterial inactivation. They are capable of inactivating bacteria, fungi, and viruses at very short application times. However, the impact on biological macromolecules is not well understood. We investigate the impact of plasma on prominent macromolecules typically found in bacterial cells to broaden the understanding of plasma inactivation mechanisms. The effects of plasma treatment on different kinds of nucleotides (double-stranded DNA, single-stranded RNA) are investigated and discussed in the context of their potential role in bacterial inactivation.**

Keywords: low-pressure plasma, bacterial inactivation, macromolecule damage

Introduction

Sterilization and decontamination are of major concern both in various industrial branches as well as in hospitals. Surgical instruments and numerous industrial products, *e.g.* laboratory equipment or food packages, have to be decontaminated and residual biological materials removed before use. Plasmas show promising potential for both applications, decontamination and cleaning, even simultaneously. Atmospheric and low-pressure plasmas can both sterilize and clean surfaces at low temperatures effectively requiring only short application times. The cleaning of surfaces by etching is a key advantage of plasma sterilization as compared to other sterilization methods.

Living organisms, including bacterial cells, consist of many different macromolecule types, the most prominent ones being DNA, RNA, and proteins. All three types of macromolecules are essential for cellular function. According to the dogma of molecular biology, genetic information stored in DNA level is transcribed into short-living RNAs. These RNAs are used as a matrix for protein synthesis (translation). Proteins are the main players in most cellular processes. Any disruption of the DNA-to-protein synthesis chain will lead to cell death. Interactions between these macromolecules and plasma might be a major inactivation mechanism of plasma sterilization. When enough macromolecules are impaired during plasma treatment, they overwhelm the cellular repair and detoxification machineries so that dysfunctional macromolecules accumulate and the cells die.

The question remains, which kind of molecular modifications are induced by plasma treatment. Plasma consists of different components, *e.g.* (V)UV radiation, radicals, and accelerated ions. These components can react in various ways with different macromolecules. We treated macromolecules *in vitro* in a low-pressure very high frequency capacitively coupled plasma (VHF-CCP) chamber. By analyzing each class

of molecules independently, we can investigate which macromolecules are damaged by plasma in which way. Furthermore, the characterization of the damage occurring different in molecule classes will allow us to deduce which kind of damage is the predominant factor in realizing plasma sterilization under defined plasma conditions.

Results

Before treating different macromolecules, inactivation assays were performed to measure the effects on living cells under the applied plasma conditions. The VHF-CCP reactor operating at 5 Pa (for further information, see talk "Characterization of a novel VHF-CCP for sterilization and decontamination of medical instruments" held by K. Stapelmann) was used to treat *Escherichia coli* cells for 8 to 128 seconds. Monolayers of cells were sprayed onto glass slides as described in [1] and colony forming units (CFUs) quantified via serial dilution plating. Complete inactivation was achieved after 64 seconds (Fig. 1). Therefore, in subsequent *in vitro* studies, macromolecules were treated for 8 to 60 seconds.

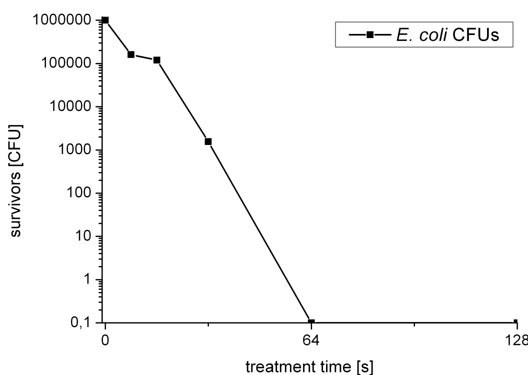

Figure 1: *E. coli* inactivation by VHF-CCP treatment

Monolayers of *E. coli* cells were treated with H_2 plasma. Serial dilution assays repeated three times were used to determine the number of survivors. The 0 second treatment time point indicates CFUs after 3 minutes of vacuum-only treatment.

Potential DNA damage was investigated using pUC18 plasmid DNA with a total size of about 2.7 kilo base pairs (kbp). After treating samples of 5 µg plasmid DNA in hydrogen plasma for up to 60 seconds on glass slides, plasmid DNA was washed off the slides with DNase-free water and analyzed by agarose gel electrophoresis (Fig. 2).

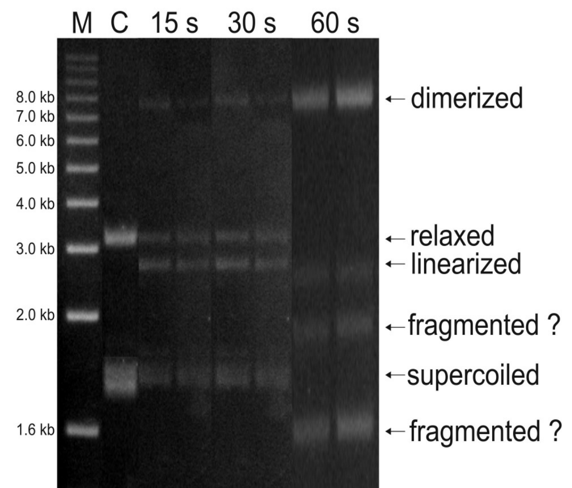

Figure 2: Plasmid DNA treated in the VHF-CCP reactor

The control DNA (C) was incubated in the reactor without plasma for 120 seconds. DNA was treated for 15, 30, or 60 seconds. After plasma treatment, DNA was analyzed by agarose gel electrophoresis (1 % agarose). The molecular weight standard (M) is a commercially available 10 kb ladder (Fermentas, St. Leon-Rot, Germany). Exposure times, brightness, and contrast were modified individually for the different test conditions to optimize band visibility.

After plasma treatment, additional band signals were observed in the gel. In all cases an additional signal at about 2.7 kbp appeared in the gel. This indicates introduction of double strand breaks into circular plasmid DNA. Furthermore, a signal at about 7 kbps appeared in the gel, with intensities increasing with treatment time. A possible explanation could be the formation of plasmid dimers. We already demonstrated formation of plasmid dimers and introduction of single strand breaks in dried DNA by (V)UV radiation emitted from an atmospheric plasma source [2]. Formation of plasmid dimers is most likely due to

crosslinking of bases from different plasmids. It was shown previously that UV radiation is capable of crosslinking DNA fragments [3]. (V)UV radiation emitted by VHF-CCP is much stronger than the (V)UV radiation emitted by the atmospheric plasma jet used in our previous experiments, hence dimer formation occurred in a shorter time frame. Additionally, DNA bands of lower molecular weight occurred at the 60 second time point. It is known that strong (V)UV radiation is also capable of producing double strand breaks in DNA [4].

Signals at about 2 kbp and 1.6 kbp most likely indicate plasmid fragmentation, where two or more double strand breaks cause the former circular structure to break into shorter fragments. These bands appear less distinct compared to the signals at lower treatment times. Most likely, strand breaking is a statistical process with the different fragments differing in size and migrating differently in the gel. These *in vitro* experiments indicate that DNA can be fragmented by high intensity (V)UV radiation under low pressure conditions. This capability is very useful for certain industrial applications, as the fragmentation and removal of DNA from *e.g.* laboratory equipment is a challenging process for the industry. It also highlights one possible cell inactivation mechanism in this reactor.

To further understand the impact of plasma on nucleotides *E. coli* total RNA was extracted from overnight cultures, 10 µg samples spotted on glass slides, dried, and treated for 16 seconds with hydrogen plasma. After treatment, RNA was washed off the glass slide with RNase-free water and analyzed by gel electrophoresis (Fig. 3). In contrast to plasmid treatment, no multimer formation could be observed. Furthermore, the fast loss of band intensity indicates that RNA is damaged by plasma treatment much faster than DNA. Most likely, the single-stranded RNA is broken down into smaller fragments or etched away. Further investigations of the molecular impact of plasma on RNA are underway.

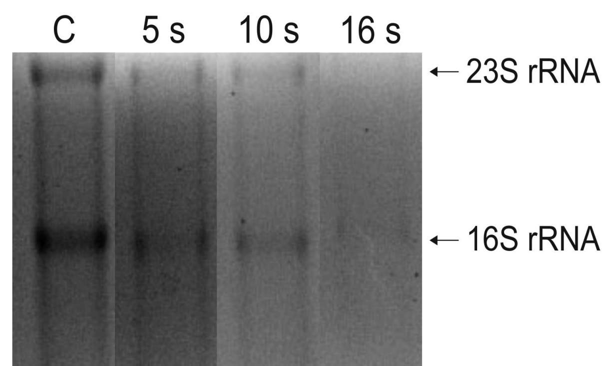

Figure 3: VHF-CCP impact on *E. coli* total RNA

Control (C) RNA was incubated in the reactor without plasma ignition for 120 seconds. Samples were incubated for 5, 10, or 16 seconds. Distinct bands correspond to 23S and 16S rRNA. Band intensity loss is observed in correlation to treatment time.

The rapid degradation of RNA by plasma offers great industrial potential, as the removal of RNA is crucial for many materials intended for laboratory use. Furthermore, rapid RNA destruction might also play a role in bacterial inactivation, as the cells need functional RNA for protein synthesis. Destruction of RNA, especially in combination with damage to other macromolecules leads to a bottleneck in production of functional proteins.

Conclusions

Low-pressure plasmas offer great potential for industrial applications. They are not only able to inactivate microorganisms, they are also able to rapidly damage and potentially inactivate different kinds of biological macromolecules. This could significantly broaden their industrial use for freeing delicate products from biological decontaminations.

References

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