Destruction of Bacillus Subtilis Cells Using an Atmospheric-Pressure Dielectric Capillary Electrode Discharge Plasma

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ABSTRACT

We report the results of experiments aimed at the investigation of the destruction of spore-forming bacteria, which are believed to be among the most resistant microorganisms, using a novel atmospheric-pressure dielectric capillary electrode discharge plasma. Various well-characterized cultures of Bacillus subtilis were prepared, subjected to atmospheric-pressure plasma jets emanating from a plasma shower reactor operated either in He or in air (N_2/O_2 mixture) at various power levels and exposure times, and analyzed after plasma treatment. Reductions in colony-forming units ranged from 10^4 (He plasma) to 10^8 (air plasma) for plasma exposure times of less than 10 minutes.

I. Introduction

The interaction of plasmas with chemical and biological agents, in particular in the context of sterilization and decontamination has received much attention in recent years [1-5]. Particular emphasis has been on the utilization of atmospheric-pressure plasmas as they do not require operation in costly vacuum enclosures and thus facilitate the convenient and low-cost treatment of large surface areas. However, atmospheric-pressure discharge plasmas are highly susceptible to instabilities and the generation and reliable maintenance of uniform, large-volume discharge plasmas at or near atmospheric pressure remain formidable challenges. A new concept to generate and maintain atmospheric-pressure plasmas over a wide range of operating conditions was developed at Stevens Institute of Technology [6,7]. The atmospheric-pressure plasma is produced using a patented capillary dielectric electrode discharge concept [7] that employs dielectric capillaries that cover one or both electrodes of the discharge reactor. The capillaries serve as plasma sources, which produce jets of high-intensity plasma at atmospheric pressure in a variety of carrier gases under the right operating conditions.

Spore-forming bacteria, in particular bacteria of the genera Bacillus, are believed to be among the most resistant microorganisms. The species Bacillus subtilis has received particular attention, as these bacteria are easy to grow in a reproducible fashion under chemically well-defined conditions. As a result, Bacillus subtilis has been the species of choice in many sterilization experiments in the past (see e.g. Refs. [8] and [9] and references therein to earlier work). Reliable and reproducible experimental data on sterilization rates have been obtained by different investigators using a variety of methods and can readily be compared.

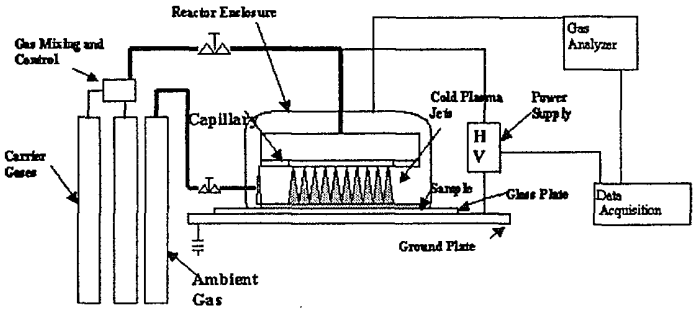
In this paper, we report the results of experiments aimed at the quantitative determination of the destruction of spore-forming bacteria using a novel atmospheric-pressure plasma shower reactor [6,7]. We established a straightforward protocol to prepare and characterize various bacteria including Bacillus subtilis on either glass or aluminum surface supports and analyze the samples after treatment by atmospheric-pressure plasma jets emanating from the plasma reactor using either in He or air (N_2/O_2 mixture) as a carrier gas at varying power levels and exposure times. We found significant reductions in colony-forming units ranging from 10^4 (He plasma) to 10^8 (air plasma) for plasma exposure times of less than 10 minutes.

II. Experimental Details

II.1 The Atmospheric-Pressure Capillary Electrode Plasma

The atmospheric-pressure plasma jets are produced using a patented capillary dielectric electrode discharge concept [6,7], which employs dielectric capillaries that cover one or both electrodes of the discharge reactor (see fig. 1). The capillaries produce jets of high-intensity plasma at atmospheric pressure under the right operating conditions. The plasma jets emerge from the end of the capillary and form a "plasma electrode" for the main discharge plasma. Under the right combination of capillary geometry, dielectric material, and exciting electric field, a steady state can be achieved. In the present study we used PlasmaSol's patented system [10] either with pure He, air (N_2/O_2 mixture), or a combination thereof as a carrier gas. The plasma jets emanating from the plasma source were fired against well-characterized cultures placed either on glass or aluminum substrates. In the course of this work we determined both the cell kill rate of the microorganism caused by the plasma exposure as well as the removal of the residual biomass from the respective substrate (cleaning).

Fig. 1: Schematic diagram of the Plasma Reactor.



II.2 Characteristics of the Micro-organisms

We used several Bacillus subtilis strains as prototypical examples of spore-forming bacteria. In some cases, we also used non-spore-forming bacteria (Pseudomonas fluorescens ATCC 1474) for selected experiments. The growth kinetics of the selected bacteria as well as the cultivation conditions that were used in the present work has been described previously [11,12]. The bacteria were sub-cultured on Difco nutrient broth slants. The spore suspensions were obtained from homogeneous batch cultures grown on chemically defined media.

III.3 Sample Preparation and Analysis

The protocol for the preparation of the samples for plasma treatment and for establishing reliable bacterial counts after the plasma treatment consists of the following steps:

(i) 100 µl of the stock spore suspension was distributed evenly across a standard microscope slide within an area of 22 x 50 mm^2 and air-dried in a laminar-flow hood. This resulted in an essentially continuous monolayer of biofilm on the slide with patches of bare glass (total surface coverage in the range from 70% to 80%). Alternatively, the suspension (200 µl in this case) was placed on aluminum substrates in an area of 50 x 50 mm^2.

(ii) The samples were then exposed to various plasma treatments.

(iii) After the plasma treatment the biomass was removed from the glass/aluminum substrate by dispensing a small amount of Tween 80 solution on the surface, removing the wet biofilm from the substrate surface, and placing it in a Petri dish. This procedure was repeated until most bacterial mass was removed from the substrate as determined by optical density measurements of the bacterial count on the substrates using direct microscopy. We routinely achieved removal rates of up to (and in some cases exceeding) 90%.

(iv) The resulting cell suspension was transferred from the Petri dish into a test tube and sonicated to disrupt cell aggregates before carrying out the following analytical steps:

- determination of the concentration of colony forming units (CFUs) by plating
- determination of the viability of cells by micro cultivation on agar films [13]
- determination of the viability of cells by staining and direct microscopy [14,15]
- determination of the total amount of solid mass and released nucleic acid by UV/VIS spectrophotometry
- determination of the total number of residual active cells by respirometry measurements

III. Results and Discussion

In a first step, we measured the UV/VIS absorption spectrum of the spore suspension before and after several minutes of plasma treatment. The UV absorption spectrum of a suspension of Bacillus subtilis shows an increase in the absorption of the plasma-treated sample below 300 nm with a local maximum around 260 nm. This is attributed to the presence of extra-cellular compounds that are released during the plasma treatment, most likely DNA, RNA, and proteins and thus indicates the destruction of the cell by the plasma. However, the increased absorption provides only a qualitative measure of the cell kill rate.

A more quantitative measure of the efficiency of the plasma treatment on the destruction of the bacteria is the rate constant, k, for cell destruction (the so-called cell "kill" rate). The kill rate k is determined from a series of experiments in which samples are exposed to the plasma for varying periods of time (under otherwise constant plasma operating conditions) and the number of destroyed cells is plotted versus the plasma exposure time. The CFU number per ml solution plotted as a function of exposure time, y(t) follows an exponential law of the form

$$(1) \qquad y(t) = y_0 \cdot e^{-(k\,t)}$$

where y_0 denotes the initial cell density at t = 0 and k is the cell kill rate. When plotted on a semi-log scale, the data fall on a straight line whose slope allows the determination of "k". It is often more convenient to introduce a cell half-life, $t_{1/2}$, which is defined as $t_{1/2} = \ln(2)/k$ and denotes the time required to destroy half the cell population for a given plasma treatment.

The cell half-life is a particularly useful quantity when one needs to determine the required plasma exposure to achieve a pre-determined sterilization rate, e.g. a cell destruction of 4 orders of magnitude (i.e. by a factor of 10,000). With our plasma shower reactor operating in air as the carrier gas, which is of particular utility in many practical applications, we achieved cell kill rates of up to 1.6 min^{-1} for glass samples corresponding to a cell half-life of $t_{1/2} = 0.43$ min and a plasma exposure time of a little more than 5 min for a cell destruction by a factor of 10,000.

Lastly, we also used the plasma shower reactor with He as a carrier case to treat both air-dried biofilms of spores and vegetative cells and bacterial suspensions on aluminum samples. Because it is in principle more difficult to generate and maintain atmospheric-pressure plasma jets that fire against a conducting substrate (as opposed to firing against the insulating glass substrate), we had to work with lower plasma currents (and consequently with lower plasma densities) in the experiments that used the aluminum substrates. As a result, we obtained lower cell kill rates under these circumstances of about 0.1 min^{-1}.

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