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To cite this article: M Laroussi *et al* 2003 *New J. Phys.* **5** 41

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Plasma interaction with microbes

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New Journal of Physics **5** (2003) 41.1–41.10 (<http://www.njp.org/>)

Received 11 February 2003

Published 30 April 2003

Abstract. The germicidal effects of a non-equilibrium atmospheric pressure plasma generated by a novel resistive barrier discharge on representatives of the two classes of bacteria (Gram-negative and Gram-positive) are discussed. The plasma exposure, while being lethal to both bacterial classes, also produced gross structural damage in the Gram-negative *E. coli* while none was observed in the more structurally robust Gram-positive *Bacillus subtilis*. An electrophysical process involving the role of the electrostatic tension on a charged body in a plasma is invoked to explain both observations. Since the efficacy of this electrophysical process depends not only on the tensile strength of the bacterial cell wall but also on its shape and texture, the need for more experimental studies, using a wide range of bacteria belonging to various morphological groups, is suggested. Ways to further test the validity of this electrophysical lysis mechanism for Gram-negative bacteria on one hand, and also to extend its operation to the more robust Gram-positive bacteria on the other, are suggested.

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1. Introduction

While dust–plasma interactions play vital roles in numerous observed phenomena in the space environment, their scope in the industrial laboratory has grown rapidly in recent times to include such diverse areas as materials processing, microelectronics, lighting and nuclear fusion (e.g. see [1, 2]). If the characterization of dust in a plasma is extended from the usual inanimate particulates to also include microbes exposed to plasmas, these interactions also encompass biotechnology. Here we make that extension and discuss some aspects of the interaction of bacteria with low temperature plasmas used in sterilization. In particular, we will discuss the relatively new technique of using non-thermal plasma discharges at or near atmospheric pressure for the purpose. The experimental procedures, both for the plasma generation and for the morphological studies of the bacteria, are presented in section 2. The observations of the gross morphological changes of representatives of the two classes of bacteria, (Gram-negative, and Gram-positive) with widely different outer cell structure, subject to the plasma discharge, are presented in section 3, followed by a theoretical discussion in section 4 and some speculations and ideas for future work in section 5.

2. Experimental procedure

2.1. Plasma generation method

Non-equilibrium atmospheric pressure plasmas can be generated using several methods. Various electrode materials and geometries as well as various means of excitation, such as microwaves, RF and ac, have been used. The experimental results presented in this paper were obtained by the use of a novel discharge, the resistive barrier discharge (RBD) [3].

The concept of the RBD is based on the dielectric barrier discharge (DBD) configuration [4]. However, instead of a dielectric, a high-resistivity (few $\Omega\text{M cm}$) material is used to cover at least one of the electrodes. The high-resistivity layer plays the role of a distributed ballast which limits the discharge current and therefore prevents arcing [3]. Specifically, the RBD consists of two planar electrodes at least one of which is covered by a layer of high-resistivity material. The two electrodes are separated by a variable gap (with a maximum size of 5 cm) where a gas mixture can be injected (see figure 1). The electrodes are driven by an ac voltage of few kilovolts (RMS) at the line frequency of 60 Hz. The applied power necessary to generate and sustain a stable discharge is in the 50–300 W range. The plasma power density is usually in the 10–300 mW cm⁻³ range. The plasma is non-equilibrium in the sense that the temperatures of the neutral species and the ions remain close to the ambient room temperature while that of the electrons is much higher (up to 5 eV). The number density of the charged particles is in the 10¹¹–10¹² cm⁻³ range. The advantage of the RBD over the DBD is the possibility to use dc power (or low-frequency ac, 60 Hz) to drive the discharge. Using helium, a large volume diffuse cold plasma can be generated at atmospheric pressure (see figure 2). Using the RBD, Richardson *et al* [5] and Laroussi *et al* [6] reported a four orders of magnitude reduction in the original concentration of vegetative *Bacillus subtilis* cells in about 10 min. They also reported that the RBD inactivated endospores of *B. subtilis*, but not as effectively as the vegetative cells. In these experiments they used a gas mixture of 97% helium–3% oxygen.

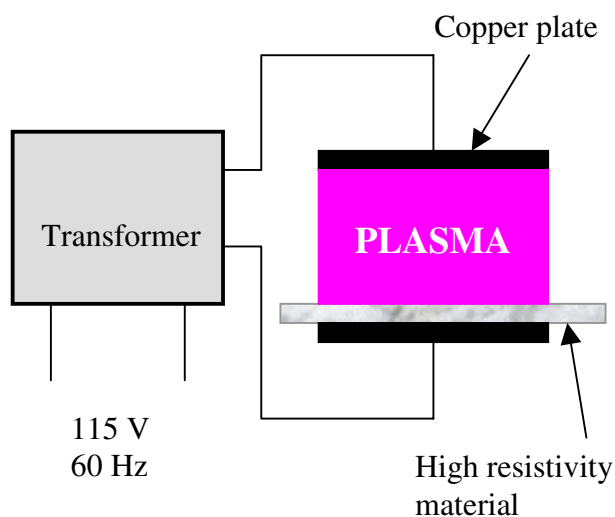


Figure 1. The RBD.

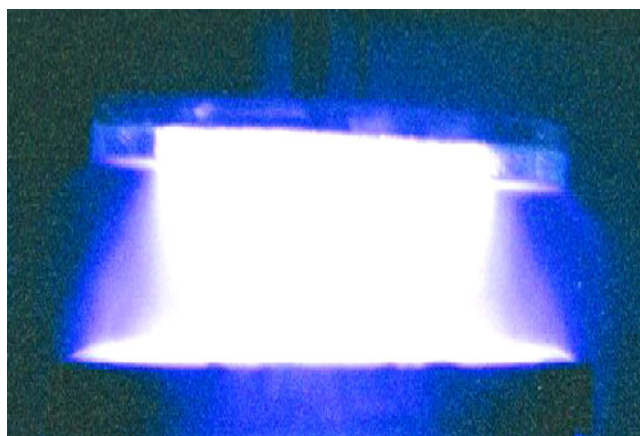


Figure 2. Photograph of the RBD. Applied voltage: 13 kV_{RMS} at 60 Hz. Electrode gap: 4.5 cm. Background gas: helium [3].

2.2. Experimental procedure for morphological studies

To investigate the effects of the plasma on the morphology of bacterial cells, scanning electron microscopy was used to inspect cells for physical damage following exposure to the plasma. Control and plasma-treated suspensions of bacteria (*E. coli*, ATCC 25922, and *B. subtilis*, ATCC 6051) were filtered onto polyester filters (13 mm diameter, pore size 0.45 μm). Filters were then placed in Teflon holders, and fixed overnight in electron microscope grade glutaraldehyde (3% final concentration in 0.1 M phosphate buffer, pH 7.2). Filter assemblies were post-fixed in osmium tetroxide (1% final concentration in 0.1 M phosphate buffer) for 1 h. Fixed preparations were then dehydrated in a graded ethanol series followed by transfer to acetone and critical-point drying. Filters were removed from the assemblies, mounted onto stubs and sputter coated with gold/palladium. Stubs were viewed with an LEO 435 VP scanning electron microscope (44 KX magnification, and 20 kV accelerating voltage). Micrographs of control and plasma-treated cells were subsequently taken for morphological inspections.

3. Observations

To investigate any effects of cold plasmas on the morphology of bacteria, micrographs obtained with scanning electron microscopy, following the procedures described earlier, were produced. Representative Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria were used in these experiments. Figure 3 shows that cells of *E. coli* were heavily damaged following exposure to the plasma, to the point where membrane integrity was largely lost and their cytoplasm was distributed in clumps on the filter. Figure 4, on the other hand, shows that cells of *B. subtilis* exhibited no morphological change relative to the control cells [7]. However, reduction in cell viability was achieved in various types of such bacteria. Cell lysis is therefore not the only mechanism whereby cells can be affected by plasmas. A possible process is the diffusion of reactive species through an otherwise chemically and physically robust outer membrane. Once inside the cell, these species can directly react with subcellular biomaterials. These reactions could either compromise the integrity of the whole cell, leading to its death, or could simply render the cell unculturable but not necessarily dead.

4. Discussion

The important observation that we focus on here is the structural damage suffered by the Gram-negative bacteria (*E. coli*) when exposed to the plasma. Their outer membrane appears to be grossly disrupted leading to the release of their cytoplasm to the surrounding medium. On the other hand, the Gram-positive bacteria (*B. subtilis*), which, unlike the Gram-negative ones, lack a thin outer membrane and possess a much thicker murein layer (providing them with higher tensile strength and rigidity), exhibit no visible morphological damage on exposure to the plasma, although they too experience a loss of cell viability. Mendis *et al* [8], referring to similar earlier observations by Laroussi *et al* [9] proposed an electrophysical mechanism for this observed structural damage of an *E. coli* bacterium, namely the electrostatic disruption of its outer cell membrane due to the electrostatic tension it experiences on being electrically charged. Here we summarize the model while using more up-to-date data on the relevant bacterial properties and a more appropriate charging assumption.

The cell envelope of the Gram-negative *E. coli* is multilayered with two membranes, an inner cytoplasmic membrane (~ 8 nm thick) and an outer lipopolysaccharide membrane (also ~ 8 nm thick) enclosing a very thin (~ 2 nm) layer of murein embedded in a gel-like periplasm layer of thickness ~ 14 nm. While the entire envelope is about 30 nm thick, it is this very thin murein layer (consisting of sugars cross-linked by amino acids) that provides the strength and rigidity of the bacterium [10, 11]. In order to simplify the calculation, while bringing out the role of the observed surface roughness of this bacterium, it was idealized as a sphere of radius R with a hemispherical irregularity (a ‘pimple’) of radius $r (\ll R)$ (see figure 5). Assuming a membrane thickness $\Delta (\ll r, R)$ and a uniform surface potential Φ , it was shown that the condition for electrostatic disruption across the base of the pimple (the more likely outcome in this case) was [8]:

$$|\Phi(V)| \geq 0.2 \sqrt{r (\mu\text{m}) R (\mu\text{m})} \sqrt{\frac{\Delta}{R}} \sqrt{F_t} \quad (1)$$

where F_t is the tensile strength of the membrane (in units of dyn cm^{-2}). If the bacterium is idealized as a perfect sphere, the condition for disruption across the middle is obtained by

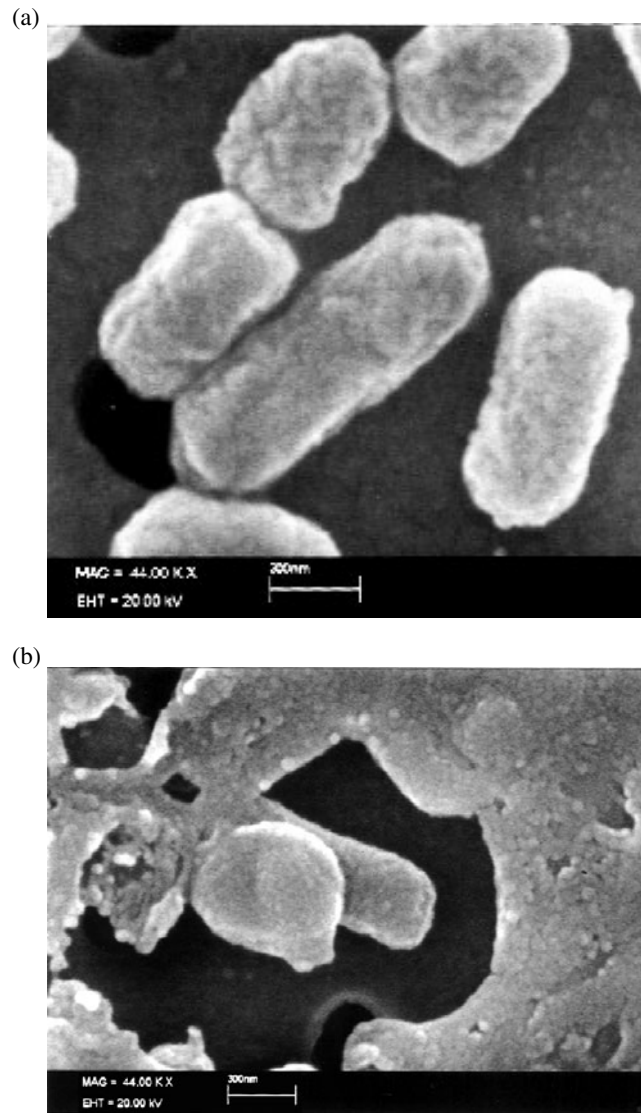


Figure 3. SEM micrographs of *E. coli* cells: (a) control; (b) plasma-treated, showing gross morphological damage [7].

replacing r by R in (1), which shows that it is easier to tear the membrane by breaking off a ‘pimple’ than by breaking into two roughly equal pieces basically because the electric field would be larger where the curvature is smaller. In the above calculation the authors estimated that $F_t \approx (1-5) \times 10^6 \text{ dyn cm}^{-2}$, based on the turgor pressures *E. coli* could support. Data that have come to our attention more recently [12] on turgor pressures (which are the internal pressures on the cell membranes resulting from the passage of H_2O across the membranes by osmosis) indicate that Gram-negative bacteria can support pressures of 3–5 atm while the more robust Gram-positive bacteria can support pressures $\gtrsim 20$ atm. So here we assume $F_t \approx (3-5) \times 10^6 \text{ dyn cm}^{-2}$ for the Gram-negative *E. coli*, and $F_t \gtrsim 2 \times 10^7 \text{ dyn cm}^{-2}$ for the Gram-positive *B. subtilis*. Assuming $R = 1 \mu\text{m}$, $r = 0.02 \mu\text{m}$ and $\Delta = 0.008 \mu\text{m}$, equation (1) leads to the condition for rupturing the ‘pimple’ to be $|\Phi| > 4.5 \text{ V}$ if $F_t \approx 3 \times 10^6 \text{ dyn cm}^{-2}$ and $|\Phi| > 6 \text{ V}$ if

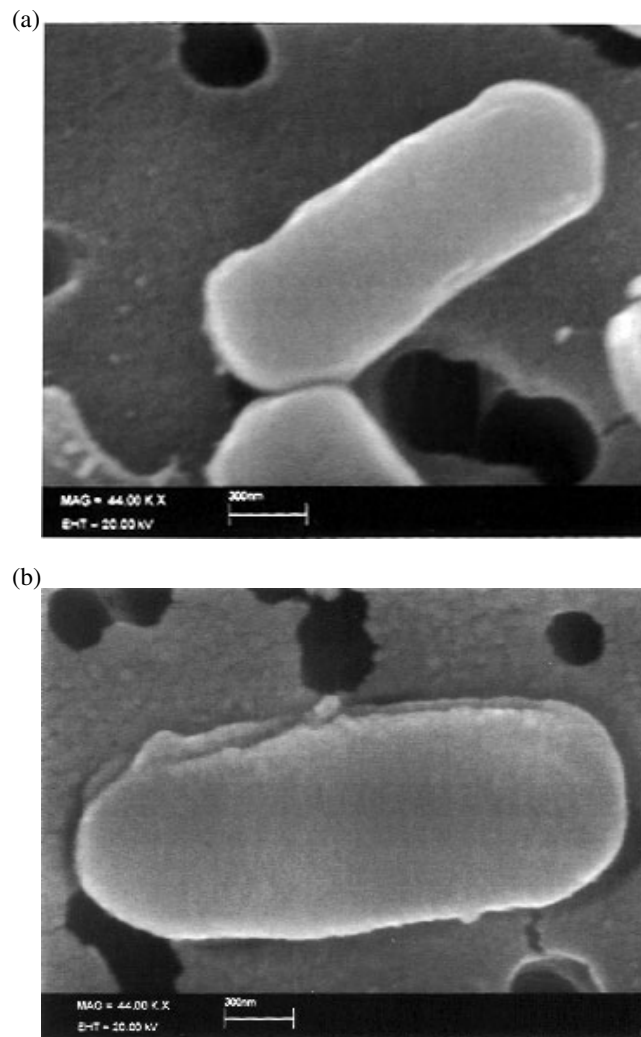


Figure 4. SEM micrographs of *B. subtilis* cells: (a) control; (b) plasma-treated, showing no apparent morphological change [7].

$F_t \approx 5 \times 10^6 \text{ dyn cm}^{-2}$. The corresponding conditions to break it in half are respectively 31 and 40 V.

If one assumes that both the electrons and ions are Maxwellian with temperatures T_e and T_i respectively (with T_e not large enough to produce secondary electron emission, as is the case here) the equilibrium surface potential Φ of any particulate immersed in it is given by the solution of the well known transcendental equation (e.g. see [13]):

$$\left(\frac{m_i}{m_e} R_T\right)^{1/2} = (1 - R_T x) \exp(-x) \quad (2)$$

where $x = e\Phi/k_B T_e$, $R_T = T_e/T_i$ and m_e , m_i are the electron and ion masses respectively. Although this equation is obtained by assuming that the collection currents are orbit limited (i.e. that the Knudsen number $Kn = \frac{\ell}{R} \gtrsim 1$, where ℓ is the collision mean-free path of the ions), it is expected that it will not be significantly changed even when $Kn < 1$, when the currents are diffusion limited (as is appropriate in the present situation). This is because in this latter case

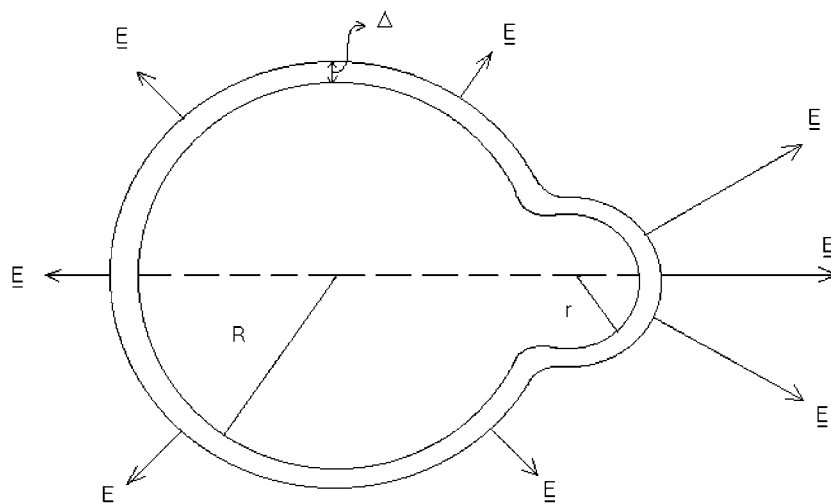


Figure 5. Simplified morphology of the *E. coli* cell envelope. Note the larger (normal) electrostatic field where the radius of curvature is smaller following from the assumption that the surface is an equipotential.

both currents are reduced by approximately the same factor, i.e. Kn [14]. The main difference is that characteristic charging time would be increased by a factor $\sim(1/Kn)$ in the diffusion limited case. In their earlier calculation Mendis *et al* [8] assumed that the discharge plasma was isothermal (i.e. $R_T = 1$). However, the ion temperature in this highly collisional atmospheric pressure plasma is close to that of the neutrals (namely room temperature). Therefore, taking $k_B T_i \approx 0.03$ eV and $k_B T_e \approx 5$ eV (i.e. $R_T \approx 167$) and solving equation (2), while noting that the ions are largely He^+ ($\sim 97\%$) we obtain $x \approx 1.5$ leading to $\Phi \approx -7.5$ V. While this surface potential on the bacterium is clearly insufficient to break the membrane in half, it is nevertheless sufficient to tear it if it has a surface roughness on the scale of a few per cent, as may be expected for the Gram-negative *E. coli* (see [11] figure 3.29(f)). We suggest this to be the cause of the observed disruption of the cell wall of *E. coli*.

We note that a necessary condition for electrostatic disruption to be the cause of the observed cell lysis of *E. coli* is that the characteristic charging time, τ , should be less than the estimated ‘kill times’ which are of the order of minutes. In fact $\tau (\approx (1/Kn)(\lambda_D/\omega_{pi})(1/R))$ is of the order of only microseconds for the condition of this experiment.

Gram-positive bacteria, such as *B. subtilis*, lack an outer membrane, but possess a thicker murein layer ($\sim 15\text{--}80$ nm) providing them with higher strength and rigidity [10]. Even if we use the smallest value of 15 nm for Δ in equation (1), while taking $F_i \approx 2.7 \times 10^7$ dyn cm^{-2} , the condition for the electrostatic rupture of a cell (assumed to be a sphere of radius 1 μm) into two more or less equal parts is $|\Phi| \gtrsim 110$ V. The surfaces of Gram-positive bacteria are observed to be generally smoother than those of the Gram-negative ones (for a comparison of the surface textures of *E. coli* and *B. subtilis*, as shown in scanning electron micrographs (see [11], figures 3.29 (e), (f)). But even if *B. subtilis* was to have a ‘pimple’ with $r \approx 0.02$ μm (as we assumed for *E. coli*) the condition for its electrostatic rupture is $|\Phi| \gtrsim 15$ V. Consequently, we do not expect to observe gross structural damage of Gram-positive bacteria such as *B. subtilis* when subject to atmospheric plasma discharges with parameters such as the one (RBD) described in this paper, and indeed this appears to be what was observed.

5. Conclusion

The plasma generated by the RBD is a source of charged particles, free radicals and radiation (IR, visible, and UV). It is believed that the synergistic effects of the charged particles, the reactive free radicals, and the radiation generated in the discharge create a lethal environment for microorganisms (e.g. [15]). However, to date, the individual roles played by each of these mechanisms are not well understood and/or quantified. The limited objective of this paper is to point out that the observed cell lysis of the Gram-negative bacteria *E. coli* might be explained in terms of the electrostatic disruption of the outer cell membrane. It is also argued that this electrophysical process will not be effective on the more robust Gram-positive bacteria for the parameters of the (RBD) plasma, consistent with the observation of non-disruption of *B. subtilis*.

Cell lysis of *E. coli* exposed to plasmas has been observed in other experiments too. For example, high-resolution transmission electron micrographs of *E. coli* subject to a uniform glow discharge plasma at atmospheric pressure also exhibited cell membrane disruption [16]. Montie *et al* [17] have proposed an alternative mechanism for the membrane rupture of *E. coli* which invokes the effects of reactive (neutral) oxygen species such as oxygen radicals produced in the glow discharge discussed in [16] on cellular macromolecules such as the membrane lipids. We suggest a way to resolve this issue. This involves using the relatively new technology of the atmospheric pressure plasma jet (APPJ), which is a non-thermal atmospheric pressure glow discharge plasma that produces a high velocity stream of highly reactive chemical species. Once the fast flowing gas exits the discharge chamber, electrons and ions are quickly lost by recombination leaving reactive radicals (e.g. O, OH) and metastable species (e.g. O₂^{*}). The APPJ has already been used to deactivate Gram-positive *Bacillus globigii* spores [18]. On using the APPJ on the Gram-negative *E. coli*, if cell lysis is observed it could support the chemical mechanism proposed by Montie *et al* [17]. If on the other hand no cell lysis is observed in this case it would suggest that a process other than the one proposed by Montie *et al* such as the electrophysical process proposed by Mendis *et al* [8], may be responsible for the cell lysis of *E. coli* observed in the RBD plasma experiment [7].

The electrostatic disruption mechanism for cell rupture described in this paper, which requires that the local electrostatic tension of the cell wall overcome its tensile strength, should be more effective as the magnitude of the surface potential increases. The magnitude of the (negative) surface potential on a particulate charged by electron and ion collection currents in a plasma increases as the mass of the plasma ions increases (see e.g. [13]). Thus we suggest that it may be easier to electrostatically disrupt a bacterium in a higher ion mass plasma. For example, if we consider a xenon (atomic mass 131.5) plasma with the same temperatures as the He plasma considered in the last section (i.e. Maxwellian electrons and ions with $T_e \sim 5$ eV and $T_i \sim 0.03$ eV), equation (2) shows that $\Phi \sim -13$ V as compared with $\Phi \sim -7.5$ V for the He plasma. It is interesting to note that the former value is near that estimated in the last section for disruption of a very small scale irregularity on a Gram-positive bacterium (where it was assumed that $r \sim 0.02$ μm and $\Delta \sim 15$ nm). Further, even larger values of $|\Phi|$ may be possible, if in addition to higher ion mass, the plasma electron distribution has a significant high-energy tail [13]. For example, if the electron distribution in the discharge is non-Maxwellian (e.g. a 'kappa' distribution with $\kappa = 2$, while the ions are Maxwellian (e.g. [13]) the surface potential could become even larger numerically $\Phi \approx -20$ V in this case). Examination of the scanning electron micrograph of *B. subtilis* (see [11] figure 3.29(e)), shows that, while its surface is smoother than highly convoluted one of *E. coli*, it does possess some small surface irregularities

(‘pimples’), which may be susceptible to electrostatic disruption if the heavier inert gas is used in the discharge. Also since the surface shape and texture appears to play such an important role in the cell lysis by this electrophysical process, we expect it to be more efficacious the more irregular the bacterial surface, as for instance appendaged and budding bacteria possessing long stalks and hyphae as well as spirochaetes and filamentous bacteria with long thin cells (see [11], figure 3.11). Clearly a systematic study of the physical consequences of the exposure to non-thermal plasmas, on a variety of bacteria from these morphological groups, could be very useful in understanding the nature of cell lysis. Another class of microbe that could be affected by this plasma interaction are bacterial endospores. While they are known to have a robust inner wall they do possess a thin delicate outer protein layer (the exosporium; [11] figure 3.64) which too could be susceptible to electrostatic lysis. Finally, viruses which are even smaller than bacteria (typically $R \sim 0.01\text{--}0.1 \mu\text{m}$) and have pronounced surface irregularities may also be expected to be highly susceptible to electrostatic structural disruption in a non-thermal plasma.

In concluding it needs to be pointed out that the concept of electrostatic disruption is not new. It has a long history going all the way back to Lord Rayleigh [19] who studied the electrostatic disruption of liquid droplets. Öpik [20] derived the basic formula for the electrostatic disruption of a uniformly conducting spherical conductor, which was later extended to non-spherical conductors (idealized as prolate spheroids) by Hill and Mendis [21]. There is a significant body of literature discussing the role of electrostatic disruption of dust in space, particularly in planetary and cometary environments (e.g. [22, 23]). In these studies the parameters that determine electrostatic disruption (besides the surface electric potential and tensile strength) are the grain size and shape. In the present work, additionally, the structure of the outer shell of the body is also seen to play a crucial role.

Acknowledgments

The work at UCSD was supported in part by DOE grant no DE-FG03-97ER54444. The work at ODU was supported by AFOSR grant no F49620-00-1-0168.

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