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Physical and biological mechanisms of direct plasma interaction with living tissue

Danil Dobrynin[1], Gregory Fridman[2,4], Gary Friedman[1] and Alexander Fridman[3]

[1] Electrical and Computer Engineering Department, College of Engineering, Drexel University, Philadelphia, PA, USA
[2] School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, USA
[3] Department of Mechanical Engineering and Mechanics, College of Engineering, Drexel University, Philadelphia, PA, USA
E-mail: gregfridman@gmail.com

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Abstract. Mechanisms of plasma interaction with living tissues and cells can be quite complex, owing to the complexity of both the plasma and the tissue. Thus, unification of all the mechanisms under one umbrella might not be possible. However, the authors are attempting to make first steps in this direction. In this paper, analysis of interaction of floating electrode dielectric barrier discharge (FE-DBD) with living tissues and cells is presented and biological and physical mechanisms are discussed. In physical mechanisms, charged species are identified as the major contributors to the desired effect and a mechanism of this interaction is proposed. Biological mechanisms are also addressed and a hypothesis of plasma selectivity and its effects is offered.

[4] Author to whom any correspondence should be addressed.

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1. Introduction

The number of potential applications of non-equilibrium atmospheric pressure discharges in biology and medicine has grown significantly in recent years [1]; in fact, the activity in this direction lead to the formation of a new field in plasma chemistry titled 'Plasma Medicine'. Some examples of medical applications of plasma are the use of plasma in the treatment of dental cavities [2, 3], sterilization of various surfaces [4]–[7], treatment of skin diseases [1, 4], [8]–[10], delicate surgeries [3, 11]–[13] and many other applications (see [1, 14, 15] for

more examples). It is now clear that these plasmas can have not only physical (e.g. burning the tissue) but also medically relevant therapeutic effects—plasmas can trigger a complex sequence of biological responses in tissues and cells. To move ahead in the further development of actual commercial tools that will enter the hospital, and in finding novel and perhaps even unexpected uses of these plasmas, an understanding of the mechanisms of interaction of non-equilibrium gas discharges with living organisms, tissues and cells becomes essential. The goal of this paper is to propose an initial model of such mechanisms.

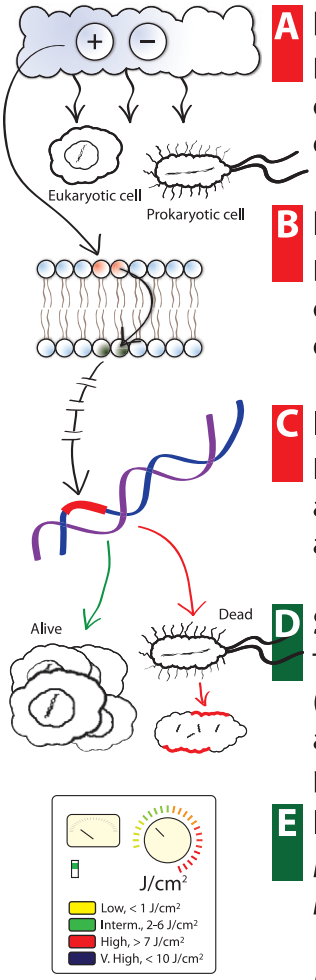
Clearly, the interaction mechanisms depend on the way the plasma is generated, the way it is delivered and the organism it is applied to; e.g. radiofrequency discharge in helium will likely have somewhat different mechanisms of interaction than afterglow from a nitrogen arc [3, 16]. Recently the authors showed that not only reactive species generated in plasma are responsible for achieving a desired effect but also the charged species (electrons and ions) are. In fact, Fridman *et al* reported that direct plasma in contact with tissue leads to a significantly faster bacteria inactivation on this tissue due to the presence of charges [17]. Below, we attempt to classify different types of species created in plasma and assess their importance in achieving bacterial inactivation and other biological effects. As the system readily available to us is the floating electrode dielectric barrier discharge (FE-DBD), we will use it as example plasma; however, we will attempt to be more general in our task of understanding the mechanisms.

2. Overview of interaction of non-equilibrium discharges with tissues, mammalian cells and bacteria

Clearly, the mechanism of interaction of plasma with living systems is complex, owing partly to the complexity of plasma and mainly to the overwhelming complexity of biology. In our current understanding, we see three distinct paradigms for plasma interaction (i) with bacteria, (ii) with mammalian cells and (iii) with tissues. In all three cases, mechanisms may and often prove to be significantly different. Further complexity is added by the presence of liquid or physisorbed water, which is always present in our treatment. Regardless of the overall complexity, in this paper, we propose a general scheme of interaction of direct non-thermal discharges with biological systems (figure 1).

To aid the reader through this paper, we begin with a general schematic summarizing the plasma–cell interactions (figure 1). In our work, we consider direct cold plasma discharge, an FE–DBD. We show that charges produced in plasma and coming in direct contact with the treatment target play a key role in the biological mechanisms through initiation and catalysis of peroxidation processes. The primary target of the direct plasma treatment is the cell membrane, the phospholipid (eukaryotes) or polysaccharide (prokaryotes) layer, where we observe all initial effects. Past the membrane, plasma-related mechanisms cease and biochemistry takes over. We show activation of complex biochemical pathways following plasma treatment, e.g. formation of malondialdehyde (MDA), which participates in the formation of DNA adducts. MDA is a good example as it is also one of the examples of *selectivity* of plasma treatment as these DNA adducts are easily repaired by mammalian cells but not by bacteria. Other examples of selectivity of plasma treatment include selective development of apoptosis in cancer cells, difference in cellular metabolism for eukaryotes and prokaryotes, etc.

We will now examine the key factors in detail.



A Plasma ions play key role

Role of positive and negative ions is related to catalysis of peroxidation processes in presence of reactive oxygen species.

B Primary target is cell membrane

Peroxidation of phospholipids and polysaccharides is catalyzed by charged species. Water is required and effect of surrounding medium is important.

C Intracellular biochemistry

Lipid layer peroxidation leads to intracellular pathway activation (i.e. malondialdehyde, MDA formation) which alters DNA structure.

D Selectivity

Tissue sterilization without visible or microscopic (histological) damage; selective development of apoptosis in cancer cells; difference in metabolism of peroxidation products; etc.

E Effect of dose

Low doses: sterilization, blood coagulation.
Intermediate doses: cell proliferation, growth factor release, apoptosis in cancers, etc.
High doses: normal cell death.
Very high doses: necrosis.

Figure 1. Summary of key findings on plasma interaction with biological organisms.

2.1. Plasma ions play a key role

We show that plasma that comes in direct contact with a biological organism achieves the desired effect orders of magnitude faster than the same dose of indirect application where plasma is separated from treatment target even by a fraction of a millimeter. We show that this effect can be primarily attributed to charged species in plasma. Thus, the key role in plasma–cell interaction is played by ions, specifically

- both positive and negative ions have relatively the same effect;
- the effect of charged species is chemical and not related to such physical phenomena as sheer stress, ion bombardment damage, or thermal effects;
- ions catalyze peroxidation processes both inside and outside of the biological organism, which explains why they are able to have greater effect than neutral active species; and
- the presence of oxygen is necessary and reactive oxygen species play a crucial intermediate role.

2.2. Primary target is the cell membrane

A careful investigation of the target of these charges reveals that most processes occur on the cell membrane, e.g. the phospholipid bilayer of a mammalian cell or the polysaccharide membrane of bacterial cell. The key processes occurring on the membrane are

- peroxidation of lipids and polysaccharides catalyzed by charges;
- the effect is chemical and highly dependent on the amount of water:
  - dry organism—low effect,
  - 'moist' organism (minute amount of non-liquid water)—highest effect, and
  - 'wet' organism (suspended in liquid)—diluted effect;
- the effect is strongly dependent on the chemical composition of the medium surrounding the cells: complete inhibition or control over the plasma effects is possible, e.g. with addition of antioxidants to the media or intracellularly.

2.3. Intracellular biochemistry

The above mechanisms are closer related to plasma than to biology as they are biochemically controlled by plasma-generated species. However, there are biological consequences of plasma treatment where plasma initiates, catalyzes, or helps sustain a complex biological response, e.g.

- a compromised membrane structure (e.g. peroxidation) or change in *membrane-bound proteins* and/or enzymes (e.g. ion channel proteins) leads to complex cell responses and may affect many cells as the affected cell signal others;
- plasma treatment may *activate intracellular signaling pathways* (e.g. turn on secondary messenger systems to amplify and transport plasma effect); and
- the initiation of subtle *secondary effects*, like the formation of MDA, which participated in DNA damage following lipid peroxidation, a process that is easily repaired by mammalian cells but not by bacteria.

2.4. Selectivity

Plasma effects can be quite selective, meaning tunable between damage to pathogenic organisms without damage to the host, or activation of different pathways in different organisms. The selectivity discussed in this paper can be summarized as

- sterilization of living tissue is achieved at two orders of magnitude lower plasma doses than required for damage, as confirmed both visually and by histological stains;
- bacteria are much smaller than mammalian cells and selectivity may be achieved simply though size/volume differences;
- biochemical differences in the organisms, e.g. polysaccharides are easier to peroxidize than lipids;
- the difference in metabolic rates between, for example, cancer cells, normal cells and bacteria may lead to difference in uptake rate of toxins generated by plasma;
- cell cycle differences: frequently replicating cancer cells and bacteria have exposed DNA compared to static mammalian cells which do not, and thus in cancers and bacteria it is easier to get to the unfolded DNA.

2.5. *Effect of dose*

The effects of dose and dose rate are quite important:

- low plasma doses ($<1\,\mathrm{J\,cm^{-2}}$): inactivation/sterilization of bacteria, normal cell survival.
- intermediate doses (2–6 $\mathrm{J\,cm^{-2}}$): repairable DNA damage, release of cell growth factors, increase in proliferation and migration, controlled development of apoptosis in cancers.
- higher doses ($>7\,\mathrm{J\,cm^{-2}}$): normal cell death.
- very high doses ($>10\,\mathrm{J\,cm^{-2}}$): cell necrosis.

This concludes the general overview of interaction of non-equilibrium discharges with tissues, mammalian cells and bacteria. In the following sections, we will discuss in detail the experimental data which lead us to formulate these hypotheses.

3. The role of charged species in direct plasma interaction with bacteria

The FE-DBD system is based on a conventional DBD and is basically a system driven by alternating current high voltage applied between two conductors, where one or both are covered with a dielectric to prevent transition to arc. In this setup, amplitude and waveform of the high voltage signal are quite important. It is widely known that damage to a surface being treated is related to the temperature of the filaments, their density and the energy per filament [18]–[21]. Uniformity of FE-DBD treatment was discussed previously and can range from a rather non-uniform continuous wave discharge to a uniform nanosecond-pulsed plasma [19, 22]. Continuous wave system can have different waveforms (sin, triangle, etc), 1–30 kHz, 10–40 kV peak-to-peak; microsecond pulse system: single polarity positive pulse, 2 μs duration, 5 $\mathrm{V\,ns^{-1}}$ rise time, 120 Hz–4 kHz pulse frequency; nanosecond pulse system: 40 ns duration, 1–3 $\mathrm{kV\,ns^{-1}}$ rise time, single pulse to 2 kHz [1, 4, 19, 20]. The continuous wave discharge has the lowest number of filaments, while operating at highest power and temperature; and this was shown to significantly damage biological tissues being treated [4, 20]. Electrical safety of the application of plasma to living tissue has been discussed in detail previously [1, 4, 15, 23]; however, toxicity of such treatment, or the extent of immediate or long-term damage, remains an open question.

There are two modes of application of FE-DBD to the surface being treated: (i) where the tissue or cells are used as a second active electrode—plasma then is bound between the dielectric surface of the powered electrode and the surface of the tissue being treated or (ii) where plasma is separated from the tissue by a grounded metal mesh and gas is blown through the discharge to carry active species outside of the plasma. We call the first method a 'direct' and the second an 'indirect' application of plasma to tissue. Authors have previously shown that direct application of plasma yields to roughly a two orders of magnitude improvement in rate of bacteria inactivation as compared to indirect application, even when the plasma is removed from the tissue by a fraction of a millimeter [17]. All the effects of direct plasma on bacteria are negligible compared to the effects of charges. In short, leaving only ultraviolet (UV) radiation (removing plasma by use of quartz (UV) or magnesium fluoride vacuum UV (VUV) windows) removes the ability of plasma to sterilize. Global gas temperature and applied electric fields are also negligible as the gas temperature rise is insufficient to achieve sterilization and the frequency and waveform utilized is out of the range for effective electroporation (see [1], [24]–[26] for further details). Effects of neutral active species cannot be ignored.

Neutrals by themselves, given enough time, are able to sterilize as well as direct plasma treatment and they are also responsible for many interesting biological effects; e.g. effect of NO in tissue regeneration [1, 10, 27]. It is important to note here that the effect of plasma is on bacteria and not on the substrate: (a) agar treated by plasma for up to 1 h remains able to grow bacteria the same way untreated agar does and bacteria appear unaffected, and (b) bacteria treated on one Petri dish and transferred to another one immediately following the treatment remain inactivated and do not grow.

3.1. Comparison of direct plasma treatment to indirect treatment with and without ion flux

In this section, three sets of experiments are discussed: comparison of direct and indirect plasma treatment of bacteria and investigation of the effect of ions of both polarities in the process of bacteria sterilization. In the case of direct plasma treatment, the discharge is ignited on the treated surface with 1.5 mm discharge gap. For indirect plasma treatment, a grounded metal mesh is used as a second electrode (22 wires per cm, 0.1 mm wire diameter, 0.35 mm openings, 60% open area, weaved mesh). The gap between the mesh and the quartz dielectric is set to 1.5 mm, just as in the case of the direct treatment setup. To ensure that both FE-DBD setups, with and without the mesh, produce the same amount of UV radiation, we correct for the UV transparency of the mesh using separate measurements. The spectrum is measured using TriVista Spectrometer System with Princeton Instruments PIMAX intensified CCD camera with and without mesh. It turns out that the mesh cuts off only about 20–40% of UV light depending on UV wavelength (figure 2).

To investigate the effect of charged species, we use agar prepared in metal dishes. These dishes are then biased with unipolar potential to extract charges from the discharge. The fact of charge extraction is confirmed by current measurements in 'dish–ground' circuit (figure 3). The plasma is ignited in the same electrode-mesh configuration as in the indirect plasma treatment experiment. Distances between the powered electrode and the mesh, and between the mesh and the agar[5], both are set to 1.5 mm.

It should be noted that in these experiments the bacteria are dried on a hydrogel surface in room conditions (∼60% relative humidity) before treatment. In such a case, bacteria are never completely dry and are covered by a minute amount of water, a condition that we refer to as 'moist'. The results of direct, indirect and bias experiments are shown in figure 4. When *Escherichia coli* are treated with plasma directly they are exposed simultaneously to charged particles, UV and all active plasma components such as ozone (O_3), hydroxyl radicals (OH) and other excited molecular and atomic species, and thus maximum inactivation effect is obtained in this case. The results of the indirect plasma treatment show that it is significantly less effective, probably due to the absence of charged species in plasma afterglow as was previously reported with skin flora (mix of staphylococci, streptococci and yeast) [17]. Applying bias potential to the agar leads to an increase in inactivation efficiency. These results show that the presence of charged species may lead to a significant increase of plasma treatment efficiency. The reader here is cautioned that although in figure 4 it may appear that there is some observable difference between positive and negative bias, we did not observe this statistically. Careful quantitative analysis of these results is underway.

[5] Note that both the dish and the agar are conductive.

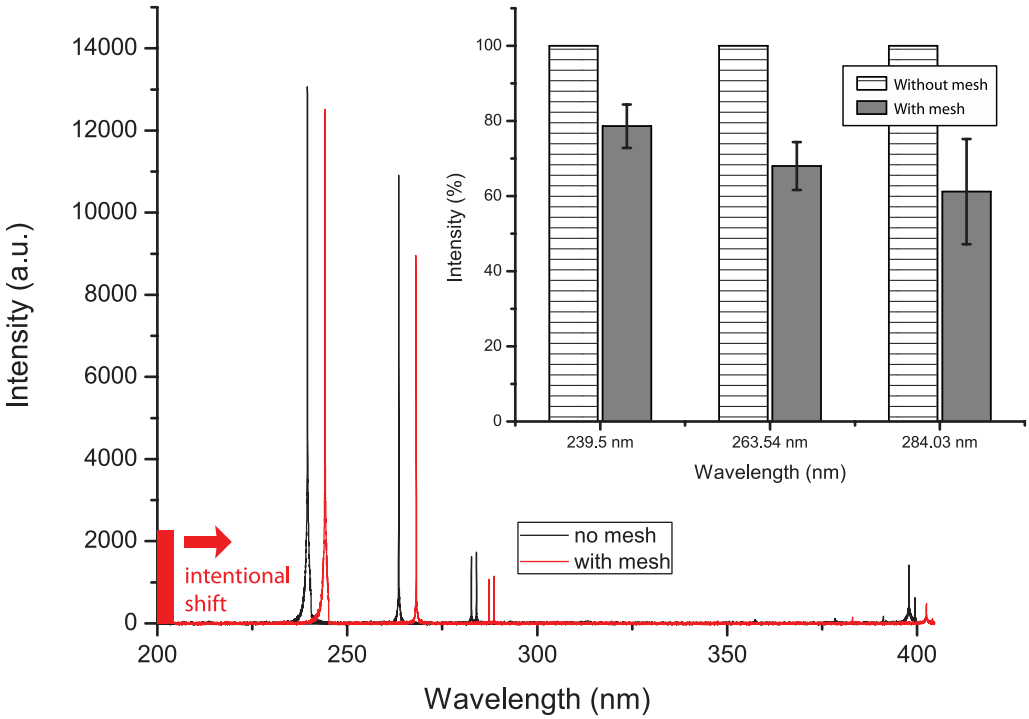


Figure 2. Results of measurement of light intensity from FE-DBD in the UV spectrum measured at three peaks (239.5, 263.54 and 284.03 nm) without mesh (taken as 100% for each wavelength) and with mesh: representative spectra and averaged data for the three peaks.

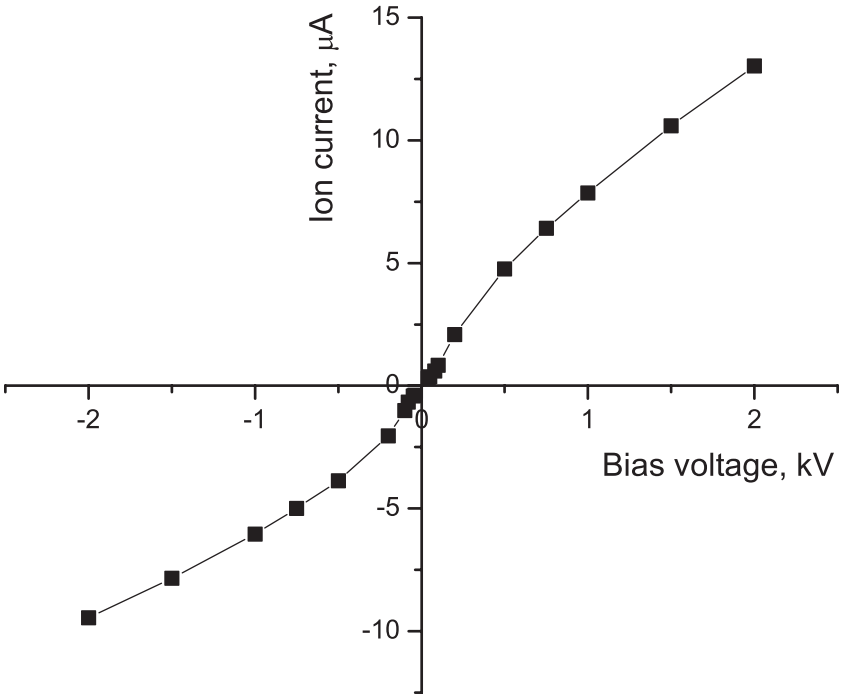


Figure 3. The results of current measurements in experiments with ion flow.

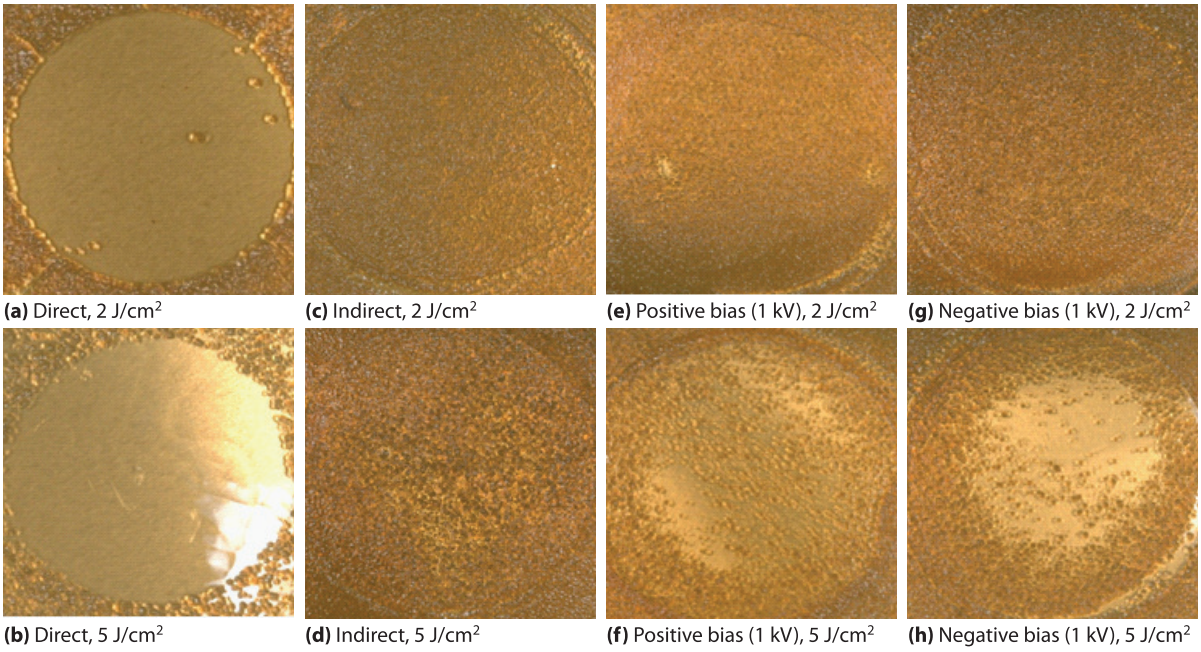


Figure 4. Results of inactivation of *E. coli* on the agar surface by direct (a, b) and indirect (c–h) plasma treatment. In the case of indirect treatment the agar was either grounded (c, d) or dc-biased with 1 kV positive polarity[6] (e, f) or 1 kV negative polarity (g, h). For all cases, the plasma dose was kept at 2 (a, c, e, g) and 5 J cm^{-2} (b, d, f, h).

3.2. The effect of positive and negative ions on inactivation of E. coli *in nitrogen corona discharge*

To study the role of ions alone, we use dc corona discharge in flow of dry nitrogen at 0.5 slpm. A stainless steel needle electrode is placed inside of ceramic tube with inner diameter of 5 mm and is powered through a 10 MΩ resistor to produce ion flow with average current of approximately 20 μA for both positive and negative polarities; voltage is then varied around 1 kV to produce the same current for both polarities. Grounded metal dish of 60 mm diameter with agar and *E. coli* is then used as a second grounded electrode, completing the circuit. There is also a 2 mm gap between end of the tube and agar surface to provide gas output from the system. Nitrogen (99.999% pure) is used to ensure that concentration of reactive oxygen species (ROS) is reduced in the discharge. The results show that ions of both polarities are able to inactivate bacteria. The effect becomes visible after ∼25 s of treatment for both cases and positive ions show slightly higher efficiency; however, the difference between positive and negative ions is no more than ∼10–15% (figure 5). No water evaporation was observed for treatment times below 1 h and the effect of gas flow alone was analyzed and shown to have no effect on bacteria (results not shown, but see [17] for similar experiment). This experiment serves as a second indication of the potential importance of ions in the inactivation of bacteria; however, it does not completely eliminate the role of UV and ROS since even in high purity Nitrogen minute amounts of water and oxygen are present.

[6] At bias voltages higher than 1 kV, we observe corona and then arc/spark discharge between mesh and agar in the control experiments; thus higher bias voltages were not used.

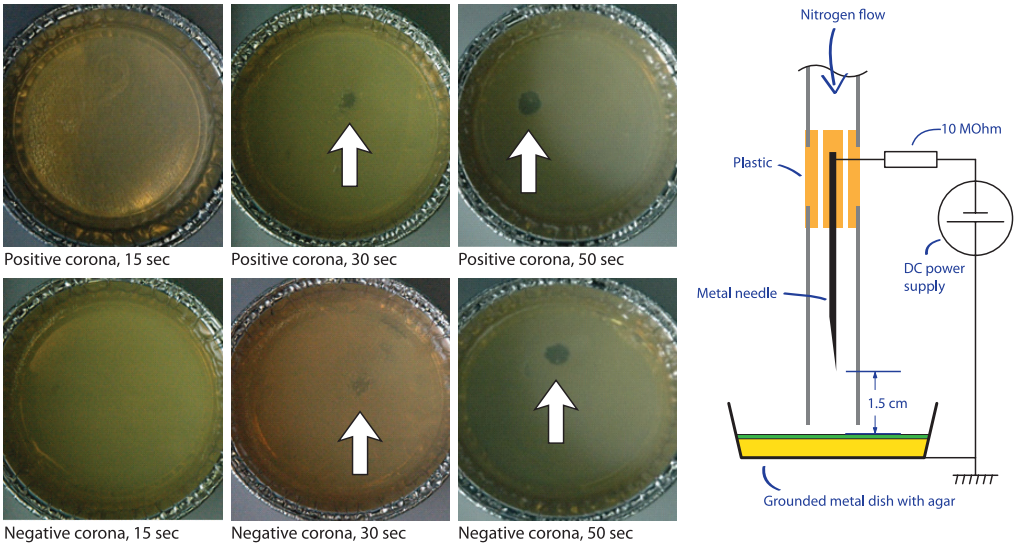


Figure 5. Schematic and results of *E. coli* inactivation on the agar surface by negative and positive polarity corona discharge in nitrogen.

4. The role of UV light, hydrogen peroxide and ozone on plasma inactivation

4.1. The effect of UV radiation in bacteria inactivation by direct DBD

The mechanism of UV-based sterilization is widely studied, and indeed in many cases it plays a major role [28, 29], therefore experiment that analyzes the role of UV radiation produced by direct plasma was carried out. One way to protect bacteria from everything that is generated in plasma, except for the UV photons, is to place a quartz glass on top of the treated surface. In our experiments, we have used quartz, which is transparent to UV photons of > 200 nm wavelengths, and MgF_2, which is transparent to VUV photons of >140 nm wavelengths [21]. This way only UV/VUV photons generated in plasma reach bacteria. As can be seen in figure 6, bacteria that are protected from direct discharge by a 10 mm square MgF_2 slide are unaffected (highest dose used was 600 J cm^{-2} with no observed difference between untreated bacteria and MgF_2-protected bacteria); thus, we can conclude that the action of UV radiation for our case can be neglected. However, even though we observe no visible effect on bacteria by UV/VUV photons, it should not be discounted completely as UV, especially VUV, is known for its synergetic effect in interaction and destruction of model polymers [18, 21, 30].

4.2. The effect of hydrogen peroxide produced by plasma on bacteria inactivation

In the presence of water molecules in room air, a certain amount of OH molecules is produced in DBD plasma. Although this highly reactive radical probably does not play a key role in the inactivation process by itself, as was discussed above, the product of recombination of two polar OH molecules on charge centers, a hydrogen peroxide molecule, may relatively easy pass cell membrane and later cause lethal effects (for example, fatal DNA damage [31, 32]). The ability of hydrogen peroxide to sterilize is widely used and well studied, and therefore we carried out an experiment that helps analyze the role of hydrogen peroxide in DBD

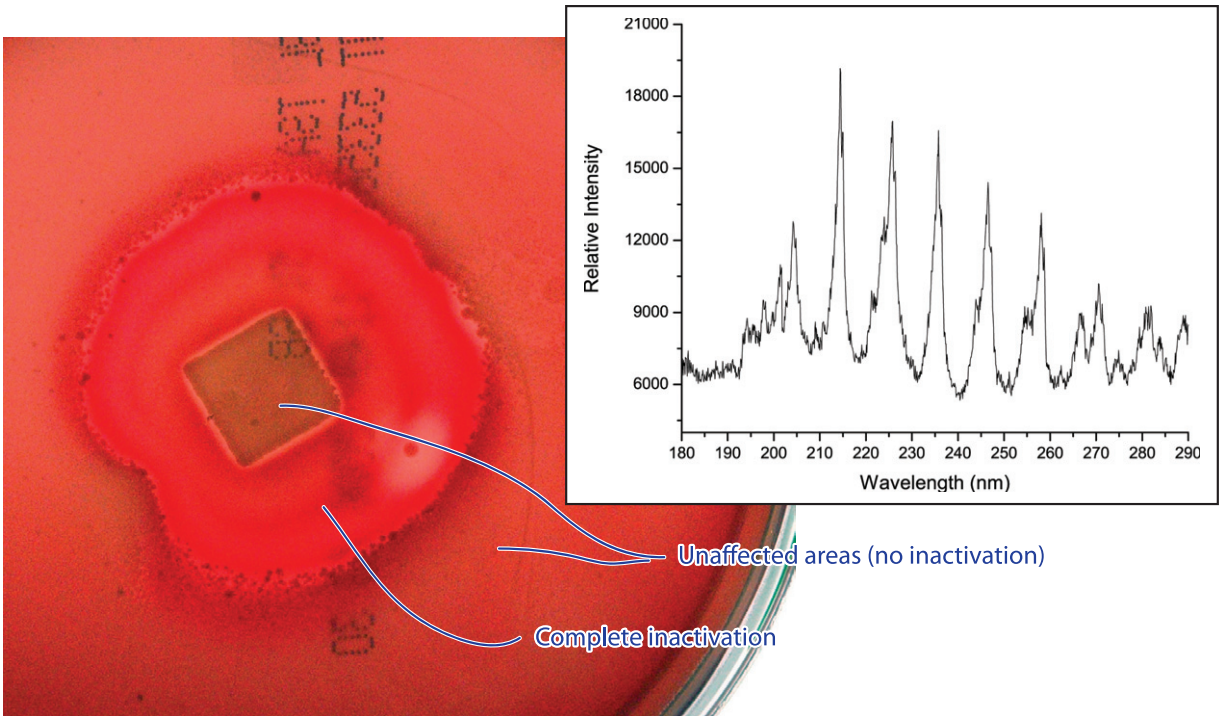


Figure 6. Emission from DBD plasma over the cell surface in the UV range (right) and results of inactivation of bacteria by direct plasma contact compared with only UV (left). No effect on bacteria protected from plasma by MgF_2 slide (10 mm square in the center) is observed. In this image, the plasma surface power density was 0.6 W cm^{-2} and the treatment dose was 108 J cm^{-2}.

plasma inactivation of bacteria. Because measurement of H_2O_2 concentration on the surface of agar is a challenging problem, we estimate this concentration using approximation of the dependence of measured amount of H_2O_2 produced in liquid. Concentration of H_2O_2 was measured in distilled water and phosphate buffered saline (PBS) with H_2O_2 specific test strips (Emd Chemicals, No.: 10081/1). Dependence of peroxide concentration on treated volume is almost linear, and increases approximately three times when the amount of treated liquid is decreased 10 times (figure 7). The estimated amount of liquid under the electrode (surface area of $\sim$5 cm^2) in the case of 'moist' agar is a few microliters; therefore, we can expect the concentration of peroxide is of the order of a few tens of mmol l^{-1} for plasma dose of several J cm^{-2}.

To determine the concentration of H_2O_2 that causes the same sterilization effect on agar in the direct plasma treatment, we use 50 volume per cent water solution of H_2O_2, further diluted with distilled de-ionized water. The 0.1 ml droplet of the solution is poured onto bacteria dried on agar and spread over the whole agar surface. The results show that concentration of H_2O_2 that corresponds to 0.5 J cm^{-2} of direct DBD plasma is more than 200 mmol l^{-1} (table 1). As can be seen from figure 7, in the best case direct DBD plasma produces 6.5 mmol l^{-1} H_2O_2 at plasma dose more than an order of magnitude higher than that required for inactivation by plasma; thus, we conclude that while hydrogen peroxide may have some effect it is not the key mechanisms by which direct plasma inactivation occurs.

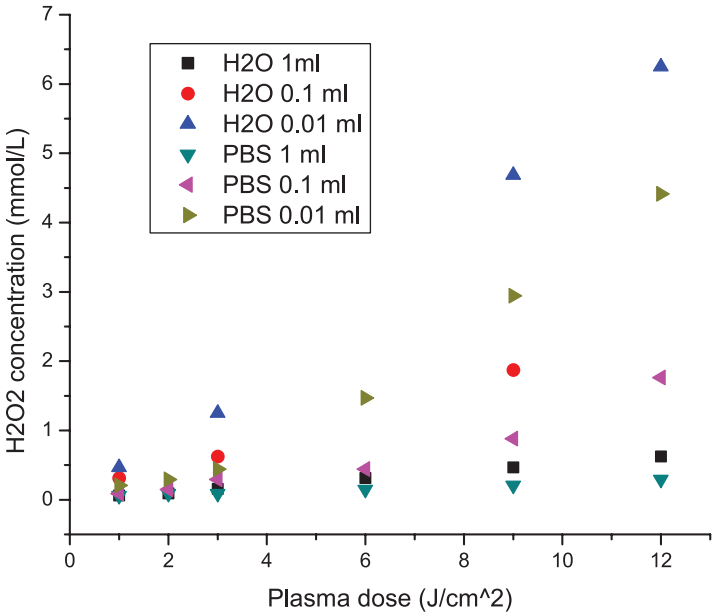


Figure 7. The dependence of peroxide concentration on volume of liquid for water and PBS.

Table 1. The effect of hydrogen peroxide on inactivation of *E. coli.*

H_2O_2 volume %	H_2O_2 mmol l^{-1}	Inactivation result
50	20 000	Complete inactivation
5	2000	
0.5	200	Some visible disinfection
5×10^{-2}	20	No visible result
5×10^{-3}	2	
5×10^{-4}	0.2	

4.3. The effect of ozone produced in plasma on inactivation of bacteria

The bactericidal effect of ozone is well known and has already been utilized in industry for some time [33]–[35]. In room air, DBD generates ozone, which may be responsible for the observed bacterial inactivation. We assess the effect of ozone in two ways. First, DBD in room air at ∼60% relative humidity produces 28 ppm of ozone measured outside of the discharge zone (recorded by ozone-specific spectrophotometer MedOzon 254/5, MedOzon, Russia). We then use ozone generator (∼500 ppm max output, Quinta Inc.) to produce the same concentration of ozone in room air without plasma [36]–[38]. No inactivation effect was observed on *E. coli* and on skin flora (mix of streptococci, staphylococci and yeast) in as much as 30 min of treatment. However, ozone meter measures ozone concentration downstream of plasma so the concentration inside plasma may be significantly different. For this reason, we use nitric oxide

Table 2. The effect of gas composition on inactivation efficiency.

Gas	Inactivation
O_2	Sterile at 2 J cm^{-2}
Air	Sterile at 2 J cm^{-2}
N_2	Some visible disinfection at $\geqslant$12 J cm^{-2}
Ar	
He	
N_2/NO (700 ppm)	

to remove ozone [39]–[41]:

$$NO + O_3 \rightarrow NO_2^* + O_2,$$

$$NO_2^* \rightarrow NO_2 + h\nu.$$

DBD plasma in gas produced by diluting a stock 700 ppm NO in N_2 with oxygen leads to the same inactivation efficiency as when pure N_2 with O_2 mixture is used while the measured ozone concentration is zero in the first case and 28 ppm in the second. Thus, we can conclude that, as with UV and H_2O_2, ozone does not play a major role in inactivation of bacteria, at least in the case of *E. coli* or skin flora on agar surface.

5. The effect of gas composition and the role of water in inactivation of bacteria

5.1. The effect of gas composition on inactivation of bacteria

As it was shown above, ions play a key role in inactivation of bacteria by direct DBD plasma; what is still one of the open questions is which specific ions are responsible. Nitrogen, for example, is an electropositive gas and N_4^+ ions are readily formed in the discharge, while oxygen is electronegative and we can expect formation of superoxide anion O_2^-. Role of ions in DBD treatment is not yet clear and for this reason additional experiments were performed to analyze behavior of plasma and its inactivation ability in different gases. Effect of treatment was observed, as before, on *E. coli*, skin flora (mix of streptococci, staphylococci and yeast obtained from human patient samples) and *B. subtilis* spores treated by FE-DBD plasma in various gases followed by a 24-h incubation and colony counts. Gases tested were air, O_2, N_2, Ar, He and N_2/NO mixture (700 ppm NO). Complete inactivation (>7 log reduction in colony forming units) of *E. coli* was achieved in direct plasma treatment in air and oxygen at 2 J cm^{-2}; in other gases tested dose of over 12 J cm^{-2} was required to achieve any visible effect and much higher doses were needed to achieve complete inactivation (no significant effect, compared to O_2 and air, was observed in Ar or He at all, even at >600 J cm^{-2}). The results are summarized in table 2. The basic conclusion of this study is that oxygen is required for fast and effective inactivation of bacteria and thus O_2^- ions may play a more important role than positive ions; although below we will show that both positive and negative ions affect bacteria and thus the need for oxygen may extend beyond a need for negative ions.

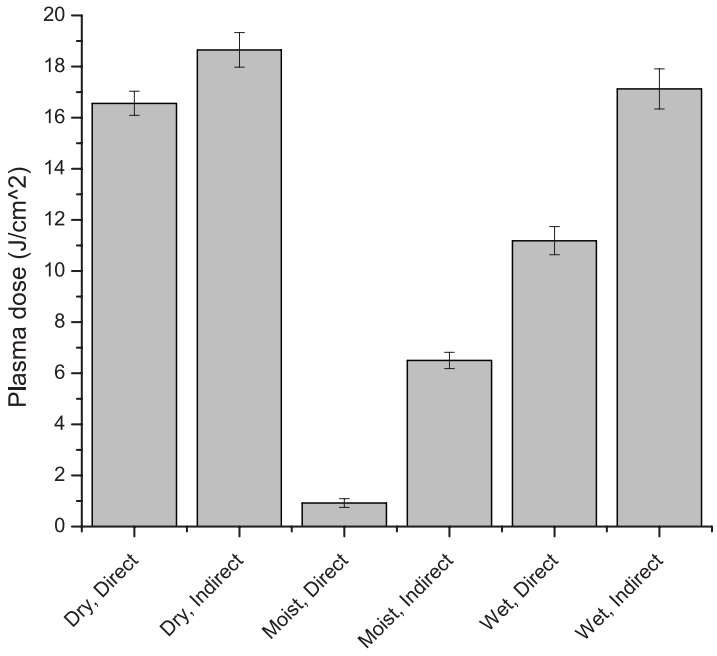


Figure 8. Results of a comparison of dry, moist and wet treatment in direct and indirect setup.

5.2. The role of water in direct plasma inactivation of bacteria

Water is present in all the plasma treatment cases discussed above. However, the effect of water and the amount required is not clear; and to analyze it we separate our treatment conditions into three groups: (a) *dry* treatment, when a droplet with bacteria is placed on a glass slide and then dried in a desiccator in a biological hood until droplet appears completely dry (∼1 h); (b) *moist* treatment, when a droplet with bacteria is placed on an agar surface and left to dry until agar appears dry (∼1 h), and (c) *wet* treatment, when a droplet with bacteria is placed in a cavity on a glass hanging drop slide and treated immediately, before water evaporates. Plasma treatment is then performed in direct or indirect conditions described above. There is a significant difference between these three cases: while on agar bacteria are covered with extremely thin, probably on the order of microns, layer of free water and the loss of charges in volume is small, in solution bacteria are covered with thick layer of water and therefore the effect of charged particles may be significantly diluted. In the case of bacteria dried on glass slides, free water is evaporated almost completely, and bacteria are covered with layer of water molecules bound by van der Waals forces. In this last case, *a priori*, the result is not clear: the effect of plasma treatment may be enhanced due to energetic ion bombardment, or may be lowered due to the lack of free water, which is required for effective oxidation processes.

Results of comparison of dry, moist and wet treatment in direct and indirect setup are shown in figure 8. For all the cases, a 0.2 ml of 10^8 CFU ml^{-1} *E. coli* solution was used and treated with plasma in ∼1 J cm^{-2} increments. It is clear that, as was previously shown by Fridman *et al* [17], direct plasma treatment achieves inactivation at lower doses than indirect. Interestingly, this remains the case for all types of treatment regardless of the amount of water. While the difference in dose required for inactivation of bacteria in moist and wet conditions

may be attributed to the amount of water protecting the organism, the case of dried bacteria is not as clear. The clear conclusion we can draw from this experiment is that the presence of water and direct plasma treatment are both required to achieve fast inactivation and this inactivation is highly dependent on the amount of water.

6. Biological effects of charged species on tissues, cells and bacteria

Radiation biology studies biological effects of ionizing and non-ionizing radiation of the whole electromagnetic spectrum. Our concern is primarily with the penetrative ionizing radiation resulting from radioactivity (alpha, beta and gamma) and x-rays. Here radiobiology claims that the main mechanism of action on a cell is through formation of ROS directly in the vicinity of a DNA molecule inside a cell nucleus [42, 43]. The ROS of interest here are hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and the superoxide anion (O_2^-). A simple stain for DNA breaks, the same test used in radiobiology, shows that the damage does, in fact, occur following plasma treatment [44]. FE-DBD plasma used in this treatment is a gas-phase phenomena and is not known to penetrate liquids, unlike radiation does. So, what is it in plasma that is able to go through liquid? Mechanism of action is apparently through the same ROS as in radiobiology; however, these ROS are not generated at the vicinity of a DNA molecule, but rather may be transported to it through a series of mechanisms already present in cells. First, a superoxide anion is generated from hydrated electrons deposited into the liquid from plasma: $e_{(H_2O)} + O_{2(H_2O)} \rightarrow O^-_{2(H_2O)}$. Mammalian cells have a mechanism of defense against superoxide and immediately convert it into less harmful hydrogen peroxide through a reaction termed 'superoxide dismutation', which is catalyzed by an enzyme appropriately termed superoxide dismutase (SOD): $2O_2^- + 2H^+ \xrightarrow{[SOD]} H_2O_2 + O_2$. Hydrogen peroxide can then travel through liquid and we do see quite high concentrations of it in liquid cell growth media (a few mmol l^{-1} as was shown above). At appropriate conditions, hydrogen peroxide is converted to hydroxyl radicals through a natural reaction chain termed Fenton mechanism (here M^+ is any ion):

$$H_2O + Fe^{2+} \rightarrow OH + OH^- + Fe^{3+},$$
$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2,$$
$$M^+ + H_2O \rightarrow M + H_2O^+,$$
$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH.$$

Hydroxyl radicals can then react with nearby organics leading to chain oxidation and thus destruction of DNA molecules as well as cellular membranes and other cell components (here R is any organic molecule):

$$OH + RH \rightarrow R\cdot + H_2O,$$
$$R\cdot + O_2 \rightarrow RO_2,$$
$$RO_2 + RH \rightarrow RO_2H + R\cdot$$

The threshold dose for DNA double strand breaks for penetrating gamma radiation of human fibroblasts is 0.5–1 Gy (J kg^{-1}) so the effective production of ROS in gamma radiation (at $\sim 10^7$ J $mole^{-1}$) is a 0.1 μM solution. An equivalent effect can be achieved by the addition of

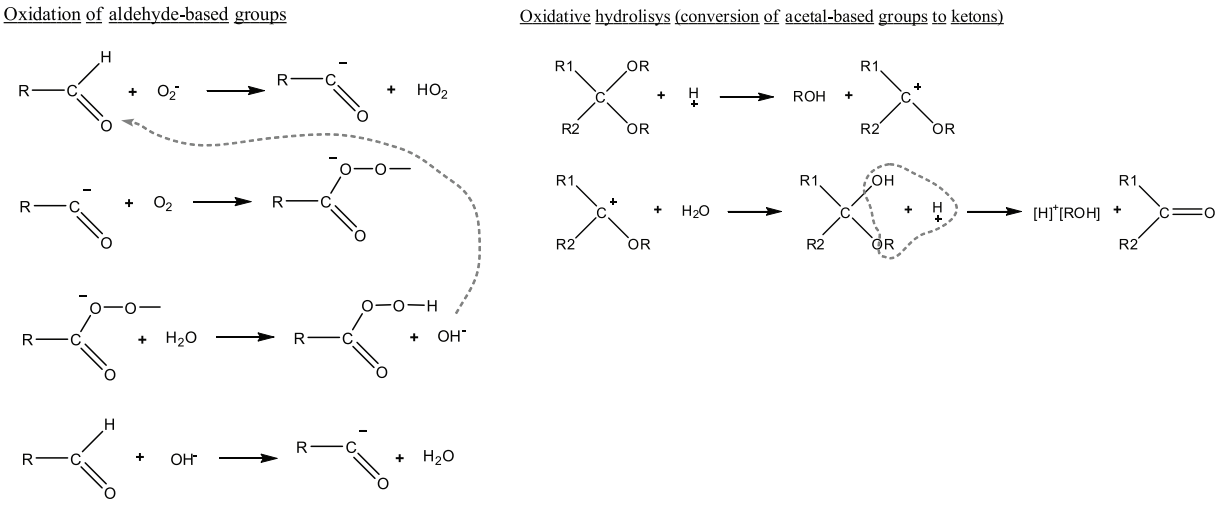


Figure 9. Oxidation reactions catalyzed by positive or negative ions that may take place in solution.

10 μM hydrogen peroxide solution outside of the cell. Thus, comparison of ionizing radiation versus H_2O_2 addition shows a 1:100 effectiveness in ROS transport through the cellular membranes to DNA. For FE-DBD plasma, threshold dose for DNA breaks in the same type of cells is 0.8 W cm^{-2}, 2 s, 2 cm^2 area: 3 J per 3 μl, or 10^6 Gy (J kg^{-1}). This means that the effective production of ROS for FE-DBD (at $\sim 10^7$ J mole^{-1}) is a 0.1 M solution, which is close to what is measured by titration assays in cell growth media treated by FE-DBD [45]. These results allow us to leverage the vast amount of knowledge generated from radiobiology studies and use it to direct our own investigations, which are clearly in need of further development.

6.1. The role of ions in chain oxidation processes

A multitude of ions may be produced in air plasma. Their concentration is lower than that of active neutral species but their effect may be stronger due to their ability to catalyze complex biochemical processes. Positive nitrogen or oxygen ions can be produced in a three-body collision: $e + 2N_2 \rightarrow N_4^+ 2e$ or $e + 2O_2 \rightarrow O_4^+ 2e$. Superoxide anion can be produced in a similar collision with another molecule, at the wall, or at a charge center: $e + O_2 + M \rightarrow O_2^- + M$. These ions are well known to create or catalyze chain oxidation processes in the presence of organic molecules; for example, oxidation of aldehydes catalyzed by negative ions or oxidative hydrolysis catalyzed by positive ions (figure 9) [46]–[48].

It is well known that these reactions can create long chains for both positive and negative ions and these chains may be much longer than the OH oxidation of organic molecules discussed in the previous section. In other words, charges may have an effect on bacteria and cells in solution due to the oxidation and peroxidation chain reactions they can catalyze; the same may be a reason for difference in doses required to inactivate bacteria versus achieve cellular damage which will be discussed in the next section.

6.2. The selectivity of plasma treatment: comparison of inactivation of bacteria to mammalian cells

A key question that still remains to be answered is the selectivity of plasma treatment, or the mechanisms by which bacteria are inactivated in just a few seconds of treatment without the apparent damage to the skin surface being treated. In this section, we will show our initial results in this direction with skin toxicity analysis on mice and pigs, DNA damage assessment, possible membrane damage mechanisms and initiation of cell growth and proliferation following plasma treatment.

6.2.1. Plasma treatment toxicity analysis on mouse and pig skin. Plasma treatment has been previously shown to be safe to skin by various research groups around the world [1, 3, 15, 23, 49, 50]. What still remains an open question is toxicity, or the extent of damage to tissue following the plasma treatment. We show that FE-DBD treatment can be applied to human cadaver skin for over 300 J cm^{-2} [4], intact mouse skin for $>$ 120 J cm^{-2} [44] and intact pig skin for $>$ 540 J cm^{-2} [51] without any visible damage analyzed by observation of skin immediately following the treatment and up to 2 weeks following treatment; and no microscopic damage analyzed by hematoxylin and eosin (H&E) staining of histological slides [1, 4, 44]. It is clear that while bacteria require a few J cm^{-2} for significant inactivation tissues remain intact at those doses; what is not clear is what happens to those tissues during and after the treatment and if there is a possibility of a long-term damage. For this reason, below we assess the extent of damage on the cellular level.

6.2.2. Reversible (repairable) DNA damage following plasma treatment. If the plasma treatment is able to penetrate tissues and cells, what is the effect it has on the cells? The effect is, indeed, non-trivial, but does seem to involve DNA. We have measured the extent of DNA damage in mammalian cells using immunofluorescence and western blot techniques with hydrogen peroxide as a positive control as it is known to induce DNA damage. Our experiments show that phosphorylation of γ-H2AX histone does occur following plasma treatment which is indicative of thymine cross-linking and DNA DSBs or DNA single strand breaks. The amount of DSBs depends on the plasma dose and it was shown that this effect is reversible for lower plasma doses (which are, interestingly, still high enough for complete inactivation of high concentration of bacteria); in other words, at plasma doses below 1 J cm^{-2}, we observe minimal effects on DNA, at higher doses of 2 to 6 J cm^{-2}, we observe DBSs but they are repaired by the cell within 24 h, and at doses above 7 J cm^{-2} amount of DNA damage is sufficient for the cell to go into apoptosis. Additionally, we show that these effects are somehow related to ROS generated in plasma as DSBs can be blocked by intracellular and extracellular anti-oxidants [52].

6.2.3. Membrane pore formation, peroxidation and damage. A likely mechanism of plasma interaction with living systems is through the membrane as it is the primary cell barrier. Many such mechanisms are possible, for example formation of small pores due to high local electric fields proposed by Schoenbach and co-workers [53, 54] may lead to either cell leakage or a pathway for entrance of toxins into the cell. These pores, depending on their size, may re-close rapidly and the cell would appear intact under the microscope while the damage done may be permanent and lead to later cell death. Another potential mechanism is through the use of the cell's own enzymes: ions present in the solution, especially ions introduced from plasma, may activate the secondary messenger system that amplifies any external signal [55]. This way,

plasma may be able to alter the behavior of the cell by simply altering the ionic strength of the solution that was previously shown with pH changes in the treated media [8, 56]. Peroxidation of phospholipid bilayer is also known to cause cellular death through a chain process leading to the formation of DNA adducts [57]–[60]. Interestingly, these defects in DNA are relatively easily repaired by mammalian cells but not by bacteria, probably due to lack of sufficient enzymes to do so [57]. In our lab we show that MDA is, in fact, released by *E. coli* following the plasma treatment at concentrations comparable to those found in the literature; this suggests that lipid peroxidation may also play an important role in inactivation of bacteria and in sub-lethal cellular effects [61].

6.2.4. Cell proliferation and release of growth factors. Oxidative stress on mammalian cells may not be as detrimental as it initially seems. Indeed, ROS induce stress in mammalian cells and while in some cases it may lead to apoptosis in other cases this stress can actually promote or control angiogenesis, the growth of new blood vessels or repair/regeneration of existing ones [62]–[64]. In our experiments, we show that plasma treatment increases proliferation of porcine aortic endothelial cells. Fold growth, determined by counting attached cells seven days following the treatment, shows a two-fold increase in proliferation compared to untreated cells. Initial results indicate that this phenomenon is due to the release of fibroblast growth factor 2 (FGF-2) by these cells as was confirmed with FGF-2 inhibitors [65, 66].

7. Biological effects of plasma on living cells

Plasma treatment can apparently have variable effects on cells. We can categorize these effects into non-damaging treatment where all cells survive, medium level treatment where cells are affected but some therapeutic effects are observed, and high doses where cell growth is impaired (figure 10) [1, 17, 52, 65, 66]. As we have shown above, effective sterilization and/or inactivation of bacteria can be achieved at doses below $2\,\mathrm{J\,cm^{-2}}$, while little effect on cells is observed; doses of 2 to $6\,\mathrm{J\,cm^{-2}}$ do have some negative but also some positive effects; and doses above $7\,\mathrm{J\,cm^{-2}}$ appear to be detrimental to cells. Below we will discuss some of the initial steps in the development of cold plasma as a medically relevant therapeutic tool with specific examples of blood coagulation, treatment of skin diseases, activation of immune response and influence on enzymatic activity of proteins.

7.1. Plasma catalysis of natural coagulation of blood and its constituents

Plasma treatment of blood can rapidly coagulate it simply by thermal desiccation, or boiling off the water [67, 68]. However, regimes exist where non-equilibrium room-temperature plasma is able to promote coagulation of blood without raising the temperature of blood or desiccating it at all [1, 4, 67]. Even more interestingly, effective FE-DBD treatment of normal whole blood, normal whole blood with sodium citrate anticoagulant and blood from a patient with Hemophilia A is clearly different [1]. This is possibly due to the mechanisms, which plasma treatment can initiate or catalyze; in other words, plasma treatment may be able to bypass the normal blood coagulation cascade and interfere directly with the later stages of the process, i.e. the previously reported effect on fibrinogen cross-linking [56].

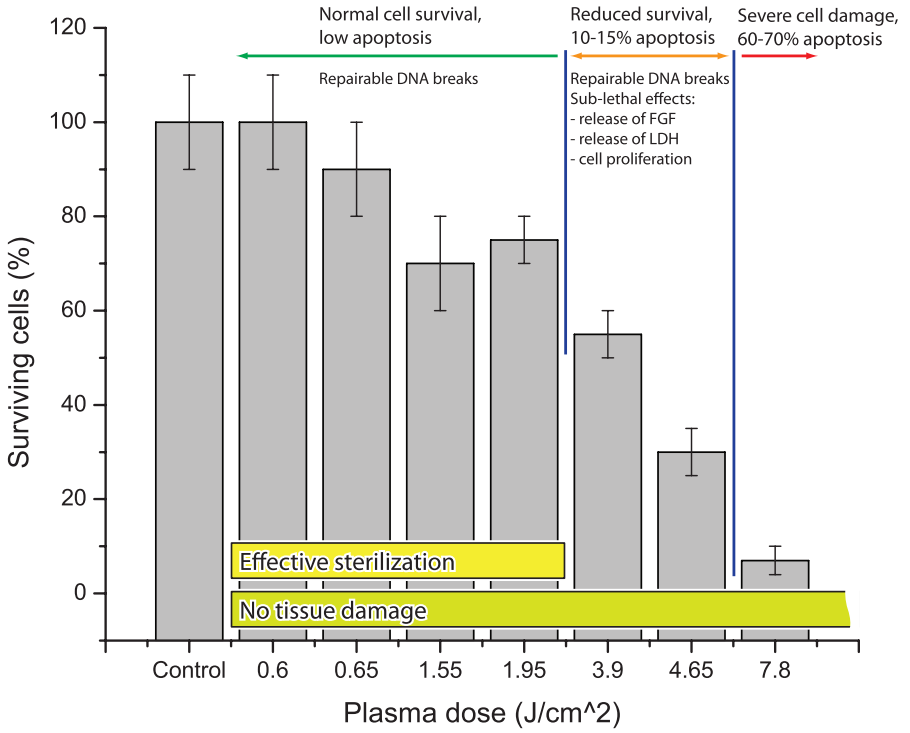


Figure 10. Summary of effects of varying doses of plasma on mammalian cells [1, 17, 52, 65, 66].

7.2. Plasma-initiated complex biochemical responses in diseased cells

We have chosen a melanoma skin cancer cell line as an example of diseased organisms. Our initial desire was to destroy these cells by plasma and that was quickly achieved [8]. A more intriguing result was obtained from the treatment of these cells in regimes where plasma caused no visible changes in the cells immediately after the treatment. The cells treated at doses below $2.5\,\mathrm{J\,cm^{-2}}$ (enough to inactivate $10^7\,\mathrm{cfu\,ml^{-1}}$ of *E. coli* [4, 69]) exhibited no changes as compared to control while they stopped proliferating and showed practically no growth 24 h following treatment. Longer than 24 h after treatment these cells began to change shape and slowly disintegrate—behavior indicative of cell self-suicide mechanism termed apoptosis. Indeed, when we stained these cells appropriately we found evidence of late apoptotic development (via TUNEL assay procedure) and early apoptosis (via Anexin V staining) [1, 8]. These results suggest that a medically relevant therapeutic effect can be achieved and complex biochemical processes can be controlled within a cell by external application of non-equilibrium plasma.

Another example of a potential application of plasma in medicine is the treatment of cutaneous leishmaniasis (CL, caused by the *Leishmania* parasite) [70]. CL results from the bite of an infected sand fly when it injects the promastigote form of the disease into the host while feeding. There the parasites are phagocytized by the host macrophages, change into amastigote form and break the host cell continuing the infection. We performed a series of *in-vitro* experiments comparing the effect of plasma on human macrophages and on the promastigote form of *Leishmania* parasite. Following a $6\,\mathrm{J\,cm^{-2}}$ dose of plasma ∼20% of

macrophages are inactivated while all promastigotes appear inactive as observed through a phase contrast microscope with trypan blue exclusion test for macrophages and simple observation for the protozoa (they stop moving the flagellum and begin to disintegrate which takes about 48 h; the organisms do not appear to re-activate following treatment).

7.3. Plasma influence on ion-channel activity in cells

Additional evidence of plasma's ability to modulate the biological responses is evident from its effects on primary B lymphocytes. These cells are central to the immune response of a host to foreign antigenic substances, including bacterial and viral pathogens. A critical intracellular messenger that triggers immune functions of B lymphocytes is the intracellular calcium concentration. Intracellular calcium is mobilized, and its cytoplasmic concentration increases when antigen interacts with the B lymphocyte Cell antigen Receptor (BCR) expressed on the surface of these cells. This interaction triggers a cascade of intracellular enzymatic reactions, including several which are sensitive to the redox state of the cells. Because ROS are reported to modulate the calcium response to antigen, we test the effects of plasma on BCR-induced calcium signals. To do this, B lymphocytes were loaded with a calcium sensitive dye (fura-2) and the intracellular calcium concentration was monitored within individual living B cells by quantitative fluorescence microscopy (represented here as the F340/F380 ratio). The cells survive plasma treatment of $> 30\,\mathrm{J\,cm^{-2}}$ without morphological changes or death. Of note here is that the cells are under a layer of flowing liquid to continually feed the nutrients required for cell operation; the liquid layer in this case is $\sim$1 mm. Moreover, plasma treatment triggers a slow increase in baseline calcium levels over time (figure 11(A)), which is blocked by pretreatment with an anti-oxidant ebselen (figure 11(B)). BCR engagement, in this case with a monoclonal antibody to the BCR, typically induces a biphasic increase in cytoplasmic calcium levels. Shortly after BCR engagement, calcium levels rise to a peak level, and then decay to a lower sustained plateau level (figure 11(C)). Interestingly, after a $30\,\mathrm{J\,cm^{-2}}$ application of plasma, this response to BCR engagement is enhanced. Both the initial peak concentration and the plateau calcium concentration are higher in plasma treated cells (figure 11(D)). These results indicate that: (i) plasma exerts effects on intracellular calcium and that anti-oxidants can suppress the effect of plasma and (ii) the response of B cells to antigen receptor engagement, and possibly the immune response, is augmented by plasma treatment [68].

7.4. Plasma influence on catalytic activity of complex proteins

The effects of plasma can apparently be selective not only to complex organisms, such as cells, but also to simpler biological compounds, namely enzymes (proteins with catalytic activity). We observe such effect, for example, on both the total protein weight of trypsin (total amount of protein in solution) and its catalytic/enzymatic activity (assessed by aFITC-Casein fluorescent microscopy protocol [1, 69, 71]) as a function of the plasma dose. The enzymatic activity of trypsin is reduced by $\sim$50% after $\sim 1\,\mathrm{J\,cm^{-2}}$ plasma dose. After $\sim 4\,\mathrm{J\,cm^{-2}}$ dose the enzymatic activity drops to zero, while the protein itself remains practically intact at treatment doses below $20\,\mathrm{J\,cm^{-2}}$ [44, 45]. This finding suggests that, at least for treatment times <1 min, plasma may be able to change the complex three-dimensional (3D) structure, and hence the catalytic activity of a protein without completely cleaving the peptide bonds and thus decimating the 2D polypeptide structure. More detailed analysis of this behavior with further results on the interaction of plasma with amino acids is being prepared by the authors.

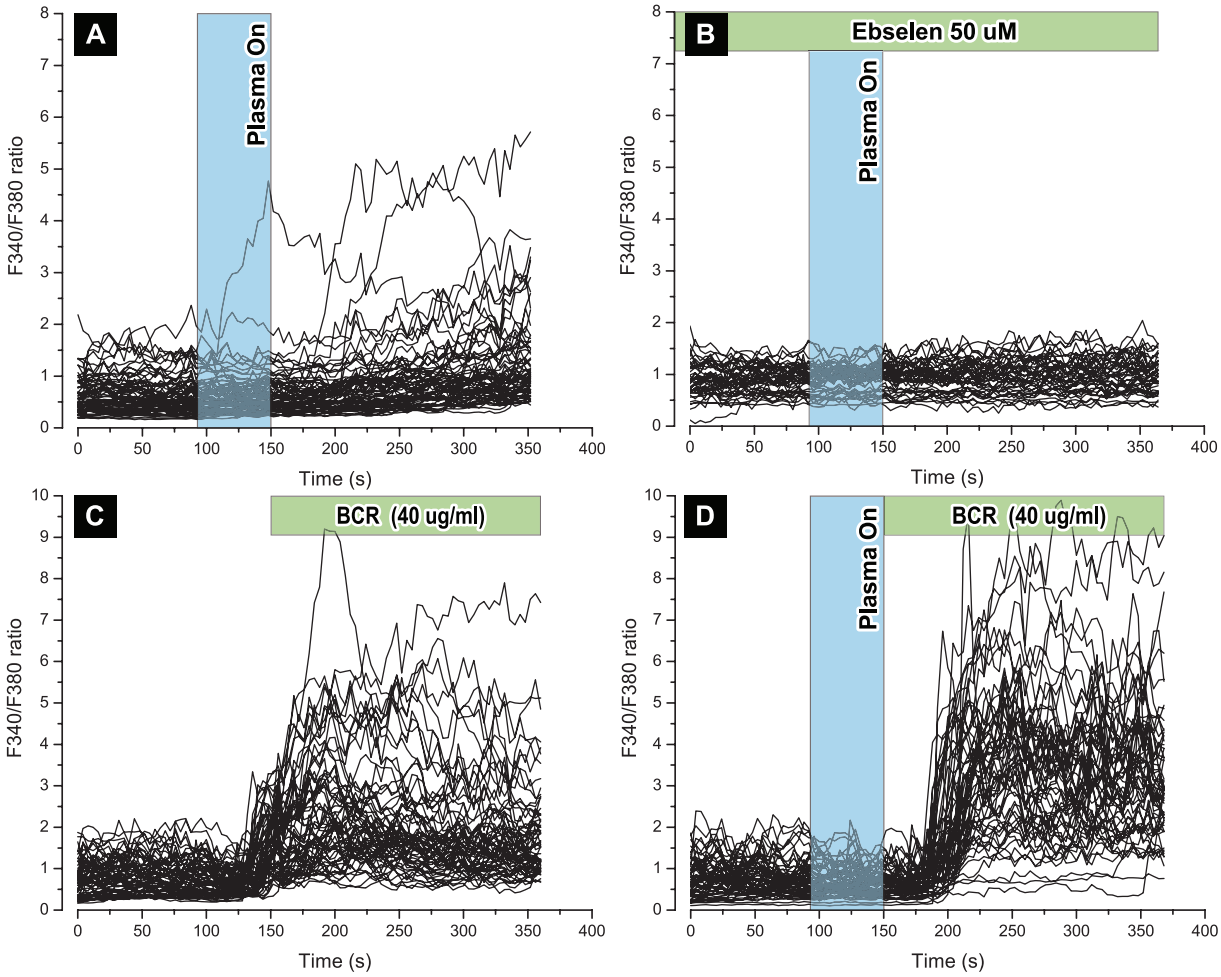


Figure 11. Results of plasma treatment of purified splenic B-lymphocyte cells. Inhibiting ROS activity suppresses the plasma effect on the steady-state calcium concentration (A and B), and antigen receptor mediated calcium signals are amplified by plasma treatment of B cells (C and D) [68].

8. Conclusion

The application of non-equilibrium plasma to cells and tissues has been shown to be selective in inactivation of pathogens, sterilization and promotion of complex biological responses with minimal or no damage to the treated tissues and cells. Based on experimental data with human cadaver tissue, living mice, living pigs and cell cultures of various mammalian cells (macrophages, epithelial cells, b-lymphocytes, fibroblasts and melanoma cancer cells) we can thus far deduce three major hypotheses for the reasons of the observed selectivity:

1. The metabolism of ROS is different in prokaryotic (bacteria) and eukaryotic (mammalian) cells; e.g. while human cells have protection from species like O_2^- bacteria either lack it completely or their resistance is lower.
2. Higher order organisms have developed more resistance mechanisms to external stress (osmotic pressure changes, ROS, chemical and biological poisons, etc); in other words,

bacteria usually act as a single cell while mammalian cells, especially those organized into tissues, communicate with each other possibly lowering the effects of the applied poison.

3. Bacterial cells are usually much smaller than mammalian cells and thus have much higher surface to volume ratio; e.g. simply a lower dose of poison is required to inactivate same number of bacteria versus mammalian cells.

These hypotheses are of course quite phenomenological and further detailed testing is required to claim them to be true. However, authors are continually receiving evidence in support of these ideas [1, 4, 8, 38, 56], [72]–[74].

Mechanisms of plasma interaction with living tissues and cells can be quite complex, owing to the complexity of both the plasma and the tissue. Thus, unification of all the mechanisms under one umbrella might not be possible. However, the authors have attempted to make first steps in this direction. Analysis of interaction of FE-DBD with living tissues and cells was presented and biological and physical mechanisms were discussed. In physical mechanisms, charged species were identified as the major contributors to the desired effect and a mechanism of this interaction was proposed, based on the existing mechanisms discussed in radiobiology where ionizing radiation is used to interact with cells through ROS generated at the vicinity of a DNA molecule. Similar mechanisms were proposed for FE-DBD plasma.

Additionally, FE-DBD plasma was shown to have biological effects on tissues and cells, a therapeutic effects that can potentially become of medical significance and lead to treatment of diseases like skin cancer, for example. Plasma treatment was shown to selectively interact with some blood constituents to promote blood coagulation through initiation or catalysis of biochemical processes pre-existing in blood. Plasma treatment was shown to control ion channel activity in cells without destroying or damaging those cells, etc.

Although these results are potentially promising, many unanswered questions and gaps in understanding remain. Authors are now attempting to focus on interaction mechanisms of plasma with live animals rather than simply cell lines in addition to more basic studies with cells. Selectivity is still an open question where deeper understanding is needed both of selectivity and of the mechanism of interaction of plasma with bacterial and parasite cells. Selectivity is apparent but its mechanisms are not fully understood, although the authors have presented three potential hypothesis.

Acknowledgments

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