Interaction of cold atmospheric plasmas with cell membranes in plasma medicine studies

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This paper discusses the mechanisms by which a low-temperature plasma produced at atmospheric pressure and low power level interacts with the cell membrane of living matter, within the context of the so-called plasma medicine. The different mechanisms of interaction with the cell membrane and cell wall in bacteria are reviewed, with considerations on the possibility of giving rise to resistance to the plasma action. The interaction with animal cells is also considered, from the perspective of exploiting the ability of the plasma to allow the insertion of large molecules, in particular genetic material, into the cells, in the so-called transfection process.

1. Introduction

The plasma medicine discipline deals with the interaction of cold plasmas produced at atmospheric pressure (cold atmospheric plasmas, or CAP) with pathogens, cells and tissues, with the aim of developing therapeutic approaches. CAP are produced at low power levels (typically of the order of a few watts dissipated over the plasma), so as to keep the treated substrate – usually a living tissue - below the temperature of 37 C, thus avoiding thermal effects. The working gas used in most plasma medicine studies is either helium or argon, with the addition of small fractions of oxygen, nitrogen, water vapour or directly air, giving rise to the formation of Reactive Oxygen and Nitrogen Species (RONS). These highly reactive chemicals are considered to be the main agent of the plasma effects on living matter, although other agents, such as charged species, electric field and ultraviolet (UV) emission may also play a role.

The seminal work on plasma medicine was carried out by Stoeffels *et al.*,[1)] who suggested the use of a CAP for the treatment of dental cavities prior to filling. Thus, this first application was focused on disinfection. Indeed, the disinfection properties of plasmas were known since many years, but the innovation of that paper was to suggest the possibility of treating living tissues instead of inanimate objects. Following this initial work, a wealth of studies has been produced, dealing not only with disinfection, but also with other therapeutic possibilities related to the capability of CAP to interact non-destructively with animal cells, triggering cellular processes. Among these possibilities one can mention wound healing[2)] and the selective killing of cancer cells.[3)]

Aim of this paper is to review the existing knowledge about the interaction of CAP with the cell membrane. In particular, the way in which the plasma action leads to the death of bacteria will be considered. Concerning eukaryotic (animal) cells, which are more resistant thanks to their defenses against oxidative stress, many possible interactions with receptors on the membrane are possible: here, a specific form of interaction will be considered, the opening of pores in the membrane, which leads to the possibility of transfecting genetic material into the cells, potentially offering an interesting alternative to the existing methods. The paper is organized as follows: section 2 gives some general concepts about the structure and function of the membrane, and of the cell wall in the case of bacteria, which are important to put into context the subsequent discussion; section 3 discusses the existing literature about how CAP interacts with cell membrane and cell wall of bacteria, ultimately leading to the plasma disinfection properties; section 4 discusses the interaction with the membrane of animal cells, with focus on poration and transfection potential; finally, in section 5 conclusions are drawn.

2. General concepts about the cell membrane

Bacteria can be divided into two large groups, Gram-positive and Gram-negative, based on the outcome of a particular type of staining, called Gram staining. The two groups differ in the structure of the cell envelope, which is the barrier separating the inner part of the cell from the outside world.[4)] Gram-negative bacteria feature three main layers in the envelope: the outer membrane, the cell wall, and the cytoplasmic or inner membrane. The two concentric membrane layers delimit an aqueous cellular compartment called periplasm. Gram-positive bacteria lack the outer membrane, but have a much thicker cell wall. Examples of Gram-positive bacteria often used in microbiological studies are *Staphilococcus aureus* and *Bacillus subtilis*, examples of Gram-negative ones are *Escherichia coli* and *Pseudomonas aeruginosa*.

The inner membrane is, like other biological membranes including the cell membrane of animal cell, a phospholipid bilayer about 8 nm thick. It is made of glycerophospholipids, molecules which feature a hydrophilic head and a hydrophobic tail. The hydrophilic head consists of a molecule of glycerol to which a phosphate group is attached. The tail is composed of two long fatty acids, which can be saturated or unsaturated, depending on the presence of double bonds along the chain. In an aqueous environment the tails are repelled, and orient themselves towards the inner part of the bilayer, whereas the heads are exposed to the outer environment or to the inner part of the cell. This is not a rigid arrangement, as the membrane has been estimated to have the viscosity of a light oil, but is able to effectively control the movement of substances in and out of cells. Embedded in the

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bilayer or attached to it are proteins, which perform a rich set of different functions, including ion channels and transporters, which are responsible for the transport of ions and molecules in and out of the cell, receptors, which sense the presence of specific molecules in the outside environment and trigger specific cell processes, and proteins responsible for the attachment of cells among themselves and to substrates.

Being the membrane relatively fluid, cell rigidity and resistance against turgor pressure is given in bacteria by the cell wall, composed of peptidoglycan, also known as murein. This is a polymeric structure made of chains consisting of alternating residues of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). Attached to the N-acetylmuramic acid is a peptide chain of three to five amino acids. The peptide chain is cross-linked by a five glycins bridge to the peptide chain of another strand, forming the 3D mesh-like layer.

The outer membrane, present only in Gram-negative bacteria, is also a lipid bilayer, but not a phospholipid bilayer. It does contain phospholipids, but they are confined to the inner side of this membrane. The outer side is composed of glycolipids, principally lipopolysaccharide.

Animal cells also have a cytoplasmic membrane, composed of a phospholipid bilayer. The main difference from that of bacteria is that eukaryotic cell membrane contains sterols, while bacterial cell membrane contains opanoids: these molecules regulate the membrane fluidity and can play a role in its permeability. Also, it is worth mentioning that animal cells are not equipped with a cell wall, with the rigidity function being performed by the cytoskeleton, a network of interlinking protein filaments present in the cytoplasm. Animal cells, being eukaryotic cells, have a nucleus and other organelles, each enclosed by its own membrane.

3. Interaction of CAP with cell membrane and cell wall in bacteria

While the destructive effect of CAP on bacteria is well known, even before the advent of plasma medicine, it is still not definitely ascertained which is the main mechanism leading to the disinfection effect. Among those which have been considered are the peroxidation of membrane lipids, the denaturation of membrane proteins, the electrostatic disruption of the cell envelope, and the effect on internal mechanisms due to the entrance in the cell of chemical species originating from outside, which can take place due to membrane poration associated to one or more of the previous effects or to the action of transporters.

Reactive oxygen radicals produced within the plasma can interact with the glycerophospholipids making up the cell membrane through lipid peroxidation. Lipid peroxidation takes place mainly with polyunsaturated fatty acids, because they contain multiple double bonds featuring especially reactive hydrogen atoms. The reaction is a chain reaction consisting of three main steps: initiation, propagation, and termination. Taking as an example the OH radical, the initiation step consists in the OH stealing a hydrogen atom from an unsaturated fatty acid, becoming H_2O and producing a fatty acid radical. The fatty acid radical is unstable, so that in the propagation phase it reacts with molecular oxygen, creating a peroxyl-fatty acid radical. This radical is also unstable, and reacts with another fatty acid chain, stealing a hydrogen atom: the result is a lipid peroxide, and another fatty acid radical, which perpetuates the chain reaction. The termination takes place when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough that the probability of collision of two radicals becomes significant. The end products of lipid peroxidation are reactive aldehydes, the most important of which is malondialdehyde (MDA). The concentration of MDA can be taken as an indicator of the amount of lipid peroxidation.

Several authors have reported an increase of MDA concentration in the treatment of bacteria with plasma. For example, Alkawareek *et al.*, using a Dielectric Barrier Discharge (DBD) jet working with helium with the addition of a small fraction of oxygen and treating *E. coli*, reported a linear increase in MDA concentration with treatment time, with values of more than 2 μg/ml for a 2-minute treatment.[5] Dolezalova and Lukes also reported an almost linear increase of MDA concentration with treatment time, using the kINPen09 radiofrequency (RF) plasma jet operating in argon, again on *E. coli*.[6] The same kind of result was found by Joshi *et al.* using a Floating Electrode DBD (FE-DBD) in air on *E. coli*.[7] Machala *et al.* quantified MDA by spectrophotometry after the reaction with thiobarbituric acid (TBA) at 90-100°C, yielding an increase of concentration of thiobarbituric acid reactive substances (TBARS).[8] Using a positive DC streamer corona (SC) and a novel regime named transient spark (TS), performed in air, to treat *Salmonella typhimurium*, they found a significant enhancement of TBARS concentration. Closely related to lipid peroxidation is the nanopore formation in artificial lipid bilayer detected by atomic force microscopy by Tero *et al.*[9] Indeed, the effect was attributed to micelle desorption induced by the accumulation inside the phospholipid bilayer of fatty aldehydes resulting from lipid peroxidation process. Another effect of plasma irradiation on artifical lipid bilayer, that is the reduction in lateral lipid mobility at the solid-liquid interface, was evidenced by Suda *et al.*[10] The effect, which was found to increase with increasing plasma power, was attributed to transient active species, whereas hydrogen peroxide was not found to play a role. The temporal behaviour of electric field and reactive species at the plasma-liquid interface had been previously computationally investigated by the same group.[11]

Another possible impact of the plasma reactive species on the cell membrane is the denaturation of membrane proteins. Membrane proteins are proteins that are part of biological membranes. They include integral proteins, that are permanently anchored or part of the membrane, and peripheral proteins, that are only temporarily attached to the lipid bilayer. By altering their structure, it is possible to affect the cell functions, eventually leading to the cell death.

Digel et al.[12] have reported that the use of a positive and negative ion generator for air purification based on

the surface discharge principle on *B. subtilis* and *E. malodoratus* had a strong impact on the membrane proteins, as detected by the 2D SDS-PAGE technique. Lipid peroxidation was also detected in the exposed bacteria using thiobarbituric acid reaction. Both effects were attributed to the hydroxyl radicals, and were located strictly within the membrane, without any damage to the cytoplasm structure. Zhang et al.[13] have studied in great detail the molecular mechanism pertaining to the inactivation of *E. coli* by air discharge plasmas, by focusing on membrane protein YgaP and intracellular protein swc7. They found that plasma treatment modifies the secondary structure and tertiary structure of the proteins, but does not affect the primary structure. Only the membrane protein appeared to be affected for exposure times lower than 1 h, and the authors concluded that plasma-induced modification of the membrane proteins is the major cause of bacterial death. Kim *et al.*, using 2-D electrophoresis, demonstrated that the application of a non-thermal plasma generator operating in air to *E. coli* for a long time (80 minutes) induced denaturation of both bacterial cytoplasmic and membrane proteins;[14] among those, outer membrane protein Tsx and glutamine ABC transporter periplasmic protein.

Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Atomic Forc Microscopy (AFM) have been used by several authors to visualize the effect of the plasma action on the individual cells. One of the first reports of this kind is the paper by Laroussi *et al.*,[15] where it was shown by SEM imaging that a DBD in a mixture of air and helium caused the treated *E. coli* cells to lose their rounded shape and release internal matter. Venezia *et al.*[16] observed with TEM clear changes in the morphology of *S. aureus* exposed to a suface discharge produced in a mixture of 1% ethylene, 50% oxygen and 49% nitrogen, especially alterations of the cell envelope, while no alterations were observed on the scanning electron micrographs of spores. Montie *et al.*[17] studied with TEM *E. coli* and *S. aureus* exposed to a DBD plasma in air, observing that in *E. coli* the continuity of the cellular envelope was breached, and cellular contents were released in the surrounding medium with a 30 s of exposure, possibly leading to cell death, while in *S. aureus* a similar effect could not be observed for the same treatment time.

Cooper *et al.*[18] tested the effect of a floating electrode DBD air plasma on *Bacillus stratosphericus*, a remarkably strong bacterium, resistant to UV as well as selected antibiotics such as penicillin, vancomycin and erythromycin,[19] on dry surface: SEM images clearly revealed etching of bacterial cell envelopes. On the other hand, the same treatment under wet conditions resulted in cell elongation, without apparent damage to the cell enevelope. The same result concerning treatment on dry surface was found by the same group for *Deinococcus radiodurans*,[20] another extremophile organism. Lunov *et al.*[21] applied two different plasma sources (one operating with low voltage, and the other with high voltage) to four different bacteria species, namely *S. aureus*, *P. aeruginosa*, *E. coli* and *B. subtilis*. SEM analysis showed that destruction of bacteria depended on plasma exposure time; furthermore, Gram-negative bacteria were more susceptible to plasma-induced destruction than Gram-positive ones, in line with the widely accepted fact that Gram-negative bacteria are more susceptible to disintegration by physical/mechanical stress. Park *et al.* also demonstrated using SEM that the morphology of *S. aureus* was changed, with rupture of cell membranes and leakage of cellular components, by the application of a DBD jet using either pure argon or argon with 1% each of either oxygen or nitrogen.[22]

Pompl *et al.*[23] used AFM to visualize two *E. coli* cells before and after a treatment with a microwave plasma torch operating with argon, showing that the cell wall was ruptured and cell plasma was released to the outside. Finally, Yamaoka *et al.* revealed, by the use of high-speed AFM, alterations in the morphological dynamic of supported lipid bilayers (SLBs), taken as a model foe cell membranes, as a result of treatment with plasma-activated Ringer's lactate solution.[24] SLB islands decreased much faster in presence of the plasma-activated solution as compared with the non-activated one, and effects such as removal of lipid molecules from the edges of SLBs could be observed.

A possible explanation for the disruption of the cell envelope has been proposed by Mendis *et al.*,[25] motivated by experimental work by Laroussi *et al.*[15] They considered that charge accumulation on envelope surface of a bacterium immersed in a plasma can lead to electrostatic forces exceeding the tensile strength required to withstand turgor pressure. By idealizing the bacterium to be essentially a sphere of radius R, with a hemispherical irregularity (a "pimple") of radius $r << R$ (indeed the cell surface is typically rough and convoluted), they evaluated that the equilibrium potential obtained by equating the orbit limited electron and ion currents to the bacterium immersed in the plasma could be sufficient to break the cell wall. More recently, Lunov *et al.*[21] have performed Stopping and Range of Ions in Matter (SRIM) numerical calculations, considering the penetration of He ions in the wall-membrane structure of Gram-positive bacteria, and in the membrane-wall-membrane structure of Gram-negative ones. They considered the effect of two different plasma sources, one operating at low voltage (725 V) and one at high voltage (4740 V), and found that for the high voltage case a relevant ion penetration inside the envelope could be found, with consequent charge accumulation in the cell. It is however worth mentioning that in typical experimental conditions the bacteria are immersed in a fluid environment (typically culture medium), so that it is questionable if such results can be directly extended. Kuwahata *et al.* described the effect of an Ar plasma jet on inactivation of *E. coli*, and suggested that the observed cell wall and membrane were disrupted by the action of electrons in the plasma jet, with excited nitrogen molecules and OH radicals playing a role in the periphery of the plasma jet.[26]

As already mentioned, the effectiveness of the plasma action for disinfection appears to be different for Gram-positive and Gram-negative bacteria. Mai-Prochnow *et al.* reported a nice graph where the number of surviving colonies was inversely correlated with the cell wall thick-

ness, for different treatment times with the kINPen argon RF plasma jet, although one should recognize that the obesrved trend mostly results from data points grouping into two groups, that is Gram-positive and Gram-negative ones.[27] Han *et al.* used a DBD in air to treat *E. coli* and *S. aureus* (Gram negative and Gram positive respectively), and found different mechanisms of action: *E. coli* was inactivated by cell envelope damage-induced leakage, while *S. aureus* was mainly eliminated by intracellular damage.[28]

It is also worth mentioning the issue of membrane depolarization. Bacteria feature a potential difference across the membrane, associated to the proton motive force, of the order of 100 mV. Cell membrane potential is indispensable for normal energy transduction and nutrient uptake in microbial cells and regarded as an important indicator for physiological activity.[29] Several authors have observed a membrane depolarization occurring as a result of the plasma treatment. Joshi *et al.* measured a dose-dependent loss of membrane potential following the treatment of E. coli by FE-DBD in air.[7] Brun *et al.*[30] found membrane depolarization to occur in *S. aureus* treated with the afterglow of a RF helium plasma,[31] and noticed it to be similar to that induced by the action of chlorhexidine, a biocide agent known to be active at the level of the cell membrane through the opening of pores. Sharkey *et al.*[32] simultaneously measured the membrane depolarization and the membrane permeabilization, and found the first effect to occur without the second, but not vice versa (both effects can of course take place together), suggesting that depolarisation of the cell membrane takes place first and is followed by (or leads to) the full permeabilization of the cell, resulting in cell death.

As we have seen, several mechanisms concur in determining the disinfectant properties of CAP. Whether one of them dominates, and which one, is crucial in defining if the plasma action can give rise to resistance, in a similar way as it happens for antibiotics. In fact, antibiotics typically target a single function or property of the cell, and resistance occurs because of mutations which allow the bacteria to counteract that specific action. The multiple targets of the plasma actions might constitute an effective way of preventing this phenomenon. Some studies have been performed in order to test the occurrence of resistance to CAP. Zimmermann *et al.*[33] treated *E. coli* and *E. mundtii* on agar plates with the HandPlaSter CAP device, based on the surface discharge concept and operating in air. The surviving bacteria were collected and cultured, and then treated again. After four such cycles no acquired resistance was observed. A similar experiment was carried out by Matthes *et al.* with *S. aureus* embedded in a biofilm, using the kINPen09 RF plasma jet in argon.[34] After six generations, spaced in time by one-hour intervals, no acquired resistance was observed. Our group also performed such an experiment,[30] using an indirect treatment deriving from the exposure to the afterglow of a RF helium plasma.[31] In this case, *P. aeruginosa* and *S. aureus* in planktonic form were tested, with six generations with 24-hour interval. Once again, no acquired resistance could be observed, whereas it could be found in similar experiments using sublethal doses of an antibiotic (ciprofloxacin for *P. aeruginosa* and daptomycin for *S. aureus*) and of a biocide agent (chlorhexidine), confirming the validity of the proposed experiment.

On the other hand, the observation of acquired resistance to the plasma action was reported by Mai-Prochnow *et al.*,[35] who considered *P. aeruginosa* cells in biofilm form surviving a double exposure to the kINPen med RF jet operating in argon. Whole genome DNA sequencing was used to perform single nucleotide polymorphism (SNP) comparison. Four of the ten detected DNA changes upon plasma exposure were found to relate to genes involved in biosynthesis of phenazines, molecules which have a protective role in oxidative stress regulation. Recently, the problem was addressed in a more systematic way by Krewing *et al.*:[36] they performed a genome-wide screening of single-gene knockout mutants of *E. coli* and identified 87 mutants that are hypersensitive to the effluent of a microscale atmospheric pressure plasma jet operated with He/O_2 as feed gas. They thus identified a set of genes that, when missing, leave the bacterial cell less well protected from plasma, which could originate acquired resistance.

4. Interaction of CAP with cell membrane in animal cells

The issue of the interaction of a low-temperature plasma with eukaryotic cells, and in particular animal cells, is even more complex than in the case of bacteria. As a general rule, eukaryotic cells are better equipped to resist oxidative stress, and can better cope with the aggression due to the reactive species produced within the plasma. Indeed, this is the origin of the extension of the plasma medicine domain beyond the realm of disinfection, since the interaction of reactive species with cell receptors can trigger complex pathways, leading to effects which range from cell proliferation and migration (useful for wound healing) to apoptosis (which can be exploited for cancer treatment). A complete overview of these interactions would be excessively long for this paper, and deserves a specific treatment. In this context, focus will be put on the possibility of exploiting the ability of the plasma to open pores in the cell membrane not in a disruptive way, as is the case for disinfection, but constructively, to allow molecules beyond the membrane barrier into the cell, and possibly up to the nucleus. This is a process which is usually named transfection, and is of relevance for several applications, mostly related to genetic engineering.

Transfection is nowadays achieved using several different methods, each having advantages and disadvantages. One widely used possibility, arguably the most related to plasma processes, is electroporation, where an electric field of appropriate intensity is able to transiently open pores in the membrane.[37] This is a simple technique, quick and with high efficiency, which however leads to a generally low cell viability, as many cells do not survive the process. A second possibility is lipofection, a chemical method in which a cationic lipid is employed to mediate the transfer of genes in the target cells.[38] This method requires no special equipment, however the

reagents are expensive and may be toxic to certain target cells. A third approach is based on the use of viral carriers to penetrate the membrane and yield gene delivery with high transfection efficiency.[39] This method can be used for *in vivo* applications, however it is associated with the risk of pathogenic expression and neoplastic transformation. A plasma treatment could offer an effective and simple non-contact and painless approach for the delivery of DNA to cells *in vivo*, without relevant cell toxicity and the risks connected to viral carriers use.

Attempts to use a plasma process for achieving transfection date from the seminal paper of Ogawa *et al.*,[40] where a pulsed arc plasma (the gas used to make the discharge was not reported) was used to transfect a green fluorescence protein plasmid into post-mitotic rat neuronal cells, human umbilical vein endothelial cells and several types of cell lines. The authors pointed out the advantage of the plasma-based method, as compared to electroporation, of being able to treat cells attached to a substrate. In a subsequent paper,[41] the transfection efficiency of a 4.7 kb plasmid (kb indicates thousands of nucleobase pairs) with a molecular weight of ~ 3.1 MDa (one Dalton corresponds to a unified atomic mass unit) into several cell lines, using the same method, was evaluated to be of the order of 20%, increasing in proportion with the plasmid DNA concentration in the medium; the cell mortality was of the order of 1%-2%. These results were confirmed by Leduc *et al.*,[42] using a helium RF afterglow jet to transfect HeLa cells with 4.8 bp hrGFP-II-1 plasmid, with a local efficiency up to 35%, without degradation of the DNA molecules if low power was used. The same group also pointed the attention to an important issue, that is the effect of the plasma on naked DNA, which can be heavily damaged, thus potentially hampering the success of the transfection process.[43]

Ramachandran *et al.* used corona discharge to produce ions deposited onto the liquid surface of culture media and demonstrated transfer of fluorescent dye calcein, drug bleomycin and nucleic acid stain SYTOX-green to murine B16 melanoma cells.[44] The first attempt of *in vivo* use of a kind of plasma treatment to achieve gene transfection can be traced to Chalberg et al.[45] They tested, as a possible therapeutic approach to retinal degenerative diseases, gene transfer to retinal pigment epithelial (RPE) cells in rabbit models, a case where conventional electroporation plus ultrasound caused severe retinal damage and did not result in efficient DNA delivery. Their approach involved an array of microelectrodes placed behind the eye, separated from the targeted RPE cells by the sclera and choroid. Microsecond biphasic voltage pulses and related current vaporise the conductive physiological medium, producing vapor bubbles which are ionized. The desired effect was achieved by the combination of electric field, chemical plasma action and mechanical stress. Connolly *et al.* tested the feasibility of using a DC helium plasma for the delivery of DNA in vivo to cells comprising murine skin, and demonstrated that non-thermal plasma can achieve significantly higher, as much as a 19-fold increase, expression when compared to injection of plasmid DNA alone, comparable to similar studies performed using electroporation.[46] The same group also showed a dose–response relationship between exposure time and the delivery of fluorescent tracer molecule to human keratinocyte cells (HaCaT) and mouse melanoma cells (B16F10) *in vitro*, with evidence that plasma exposure and electroporation affect cell membranes in a similar way.[47]

Nakajima *et al.* used a DBD plasma jet operating in argon to demonstrate that, besides bactericidal effect, there is a non-lethal plasma exposure associated to increased membrane permeability which can be exploited to achieve plasmid transfer into *E. coli*.[48] This result is also relevant for the discussion carried out in the previous section. Edelblute *et al.* used cathode-directed streamers generated by a shielded sliding discharge in air and demonstrated plasmid transfer into B16F10 cells and HaCaT cells, the latter used as a preliminary model for human skin.[49] The effect was attributed to membrane poration driven by the electric potential induced by charge accumulation on the membrane.

Xu *et al.* used a DBD plasma jet working with argon mixed with small amounts of functional gases, such as O_2, N_2 and H_2O to compare the uptake efficiencies and cell viabilities of liposome-mediated transfection and electroporation to those of plasma transfection.[50] They found that Ar or Ar + H_2O plasmas had the best efficacy, and concluded that aqueous extracellular ROS production by plasma is likely to be one of the primary factors underlying its efficacy for transfection, by increasing the endogenous ROS level. They also investigated, for the first time, the possibility of plasma transfection for delivering siRNA and miRNA in a 3D cell culture, with positive outcome, partly dependent on the molecule size.

Sasaki *et al.* used a DBD plasma jet operating in helium to transfect the fluorescent dye YOYO-1 into 3T3-L1 mouse fibroblasts, finding a double peaked curve of the transfection efficiency as a function of treatment time, with a first peak, independent of suspension volume, allowing high efficiency and high cell viability with a treatment lasting only 5 s.[51] In a subsequent paper by Kaneko *et al.*, the plasma source design was modified, with the cell solution now used as ground electrode.[52] An increased transfection efficiency was found, owing to the high density plasma generated just above the cell solution. Furthermore, an improvement was observed using adherent cells instead of floating cells, attributed to the increase in the electric field and the density of the short-lived ROS on the cells, resulting from shortening the distance between the solution surface and the adherent cells. The same group performed nice measurements of OH radicals in the gas and liquid phases, showing that these radicals are involved in membrane permeabilization.[53,54] In a further paper, they found that charged particles, superoxide anion radicals and hydrogen peroxide are strong candidate factors that play important roles in cell membrane permeabilization induced by plasma irradiation.[55] A very interesting finding of the same research group was that short lived reactive species formed in plasma-irradiated solution can trigger an influx of Ca^{2+} ions through ruthenium red- and SKF 96365-sensitive Ca^{2+}-permeable channels, possibly transient receptor potential channel family members.[56] Ca^{2+}

ions play key roles in many cellular processes, including endocytosis.

Ikeda *et al.* investigated the relationship between molecular size and transfection efficiency: molecules with low molecular mass (less than 50 kDa) and high molecular mass (more than 1MDa) were transferred to L-929 cells.[57] It was found that the transfection efficiency decreases with increasing transferred molecular size and also depends on the tertiary structure of transferred molecules.

Jinno *et al.* investigated the effectiveness of different plasma source technologies in achieving gene transfection.[58] By comparing the effect of an argon arc plasma, a helium DBD plasma jet, a helium DBD and an air "microplasma" which used a very thin capillary as high voltage electrode, they concluded that the latter was the most effective in achieving both high transfection efficiency (52.5%) and cell viability (90.8%) when used to transfect a 5.5 kb plasmid into COS-7 cells taken from African green monkey kidney. Moving from this conclusion, they tried to ascertain the relative weight of three groups of factors, i.e. electrical, chemical and biochemical ones, in determining transfection efficiency under microplasma action.[59] They found that chemical factors, while necessary for membrane permeabilization, do not work by themselves alone and electrical factors are essential to achieve plasma-mediated transfection. It was concluded that clathrin-dependent endocytosis working synergically with electroporation were the dominant factors, with neither permeation through ion channels nor chemical poration playing an important role. In a subsequent paper, they considered also other endocytosis pathways, concluding that endocytosis accounted for at least 80% of the transfer, with electrical factors playing a crucial role in determining plasmid DNA adhesion to the membrane and triggering of endocytosis.[60] Recently, they also found that when target molecules havea molecular weight lower than 10 000 bp, the transfection efficiency is proportional to the collision frequency between target molecule and cell, while when the molecular weight exceeds 10 000 bp the transfection efficiency decreases with increasing molecular weight, and almost no introduction is made at 15 000 bp or more.[61]

Vijayarangan *et al.* investigated the dependence on plasma parameters for effective drug delivery in HeLa cells using a helium DBD plasma jet fed with high voltage pulses at microsecond time scale.[62] They found that the delay between each voltage pulse is a key parameter for cell membrane permeabilization. Their conclusion was in line with that of Jinno's group, i.e. that a combined effect between plasma electric field and reactive species produced in the treated medium is necessary to ensure the effectiveness of plasma jets as an efficient drug delivery device.

The importance of the combined effect of chemical factors and electric field was also clearly shown with molecular dynamics simulations by Yusupov *et al.*[63] Simulating a phospholipid bilayer (PLB), consisting of di-oleoylphosphatidylcholine molecules covered with water layers, as a model system for the plasma membrane, they found that oxidation of the lipids results in a drop of the electric field threshold needed for pore formation in the lipid bilayer.

5. Conclusions

This short review gave a forcedly concise overview on several ways in which a cold, atmospheric pressure plasma of the kind used in plasma medicine studies interacts with the cell membrane of bacteria and animal cells. While the desired outcome of the interaction is generally different, lethal for bacteria and non-lethal for animal cells when gene transfection is sought, and despite the fact that the two kinds of cell have different properties as far as defence from oxidative stress is concerned, and on the very structure of the cell envelope, one can try to find some commonalities. First of all, there is a growing body of evidence suggesting that successful transfection in animal cells requires both the chemical effect of the reactive species produced in the plasma and a strong enough electric field. On the other hand, the two are usually considered as different possible mechanisms for cell disruption in the case of bacteria. While it is true that disinfection can be achieved also in the absence of electric field on the target, for example by exposure to the plasma afterglow, it would be worthwhile to investigate in a more thorough way if the effectiveness can be enhanced by combining the two in suitable doses. Another issue that deserves better investigation is the effect of the plasma exposure on the cell wall in bacteria, and the detailed mechanism in which the interaction between the two is taking place, at the biochemical level. Also, since at least one study has demonstrated the possibility of transiently opening pores in the membrane of bacteria at sublethal plasma doses, attention to this condition may be of great help in better elucidating the mechanisms of the plasma action on bacteria, whereas most studies primarily focus on lethal doses, being mostly interested in effective disinfection. Regarding the development of a reliable transfection methodology based on CAP, a crucial issue to be addressed is the possible damage occurring to the naked DNA molecules to be inserted into the cells due to the reactive species produced by the plasma. Some reports in this respect are already available, but the absence of such damage should be a parameter routinely used to benchmark experiments, together with transfection efficiency and cell viability. Comparative studies of the effect of different plasma sources on the same target, such as that made by Jinno et al. in the case of transfection, are also badly needed in order to discriminate among different factors (e.g. electric field presence or absence) and different forms of plasma production, which may lead to features presently not diagnosed, such as the presence of energetic ions or electrons. More generally, plasma diagnostic needs to be improved, in order to establish the full chain of links between source technology, plasma physics, chemistry and biological effects. Overall, one can conclude that much is still to be understood in relation to the processes discussed in this paper. We hope that this contribution can serve as a stimulus for discussion and further research.

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1) Stoffels E, Flikweert A J, Stoffels W W and Kroesen G M W 2002 Plasma needle: a non-destructive atmospheric plasma source for fine surface treatment of (bio)materials *Plasma Sources Sci. Technol.* **11** 383
2) Isbary G *et al.* 2013 Cold atmospheric argon plasma treatment may accelerate wound healing in chronic wounds: Results of an open retrospective randomized controlled study in vivo *Clinical Plasma Medicine* **2** 25
3) Schlegel J, Koritzer J, Boxhammer V 2013 Plasma in cancer treatment *Clinical Plasma Medicine* **2** 2
4) Silhavy T J, Kahne D and Walker S 2010 The Bacterial Cell Envelope *Cold Spring Harb Perspect Biol.* **2** a000414
5) Alkawareek M Y, Gorman S P, Graham W G and Gilmore B F 2014 Potential cellular targets and antibacterial efficacy of atmospheric pressure non-thermal plasma *Int. J. Antimicrob. Agents* **43** 154
6) Dolezalova E and Lukes P 2015 Membrane damage and active but nonculturable state in liquid cultures of Escherichia coli treated with an atmospheric pressure plasma jet *Bioelectrochemistry* **103** 7
7) Joshi S G, Cooper M, Yost A, Paff M, Ercan U K, Fridman G, Friedman G, Fridman A and Brooks A D 2011 Nonthermal dielectric-barrier discharge plasma-induced inactivation involves oxidative DNA damage and membrane lipid peroxidation in Escherichia coli *Antimicrob. Agents Chemother.* **55** 1053
8) Machala Z, Chládeková L and Pelach M 2010 Plasma agents in bio-decontamination by dc discharges in atmospheric air *J. Phys. D: Appl. Phys.* **43** 222001
9) Tero R, Yamashita R, Hashizume H, Suda Y, Takikawa H, Hori M and Ito M 2016 Nanopore formation process in artificial cell membrane induced by plasma-generated reactive oxygen species *Int. J. Arch. Biochem. Biophys.* **605** 26
10) Suda Y, Tero R, Yamashita R, Yusa K and Takikawa H 2016 Reduction in lateral lipid mobility of lipid bilayer membrane by atmospheric pressure plasma irradiation *Jpn J. Appl. Phys.* **55** 03DF05
11) Suda Y, Oda A, Kato R, Yamashita R, Tanoue H, Takikawa H and Tero R 2015 Computational study of temporal behavior of incident species impinging on a water surface in dielectric barrier discharge for the understanding of plasma–liquid interface *Jpn J. Appl. Phys.* **54** 01AF03
12) Digel I, Temiz Artmann A, Nishikawa K, Cook M, Kurulgan E and Artmann G M 2005 *Med. Biol. Eng. Comp.* **43** 800
13) Zhang H, Ma J, Shen J, Lan Y, Ding L, Qian S, Xia W, Cheng C and Chu P K 2018 Roles of membrane protein damage and intracellular protein damage in death of bacteria induced by atmospheric-pressure air discharge plasmas *RSC Adv.* **8** 21139
14) Kim Y-M, Yun H-S, Eom S-H, Sung B-J, Lee S-H, Jeon S-M, Chin S-W and Lee M-S 2018 Bactericidal Action Mechanism of Nonthermal Plasma: Denaturation of Membrane Proteins *IEEE Trans. Rad. Plasma Med. Sci.* **2** 77
15) Laroussi M, Sayler G S, Glascock B B, McCurdy B, Pearce M E, Bright N G and Maltt C M 1999 Images of biological samples undergoing sterilization by a glow discharge at atmospheric pressure *IEEE Trans. Plasma Sci.* **27**, 34
16) Venezia R A, Orrico M, Houston E, Yin S M and Naumova Y Y 2008 Lethal activity of nonthermal plasma sterilization against microorganisms *Inf. Control Hosp. Epidemiol.* **29** 430
17) Montie T C, Kelly-Wintenberg K and Roth J R 2000 An overview of research using the one atmosphere uniform glow discharge plasma (OAUGDP) for sterilization of surfaces and materials *IEEE Trans Plasma Sci.* **28** 41
18) Cooper M, Fridman G, Fridman A, Joshi S G 2010 Biological responses of Bacillus stratospheric to floating electrode-dielectric barrier discharge plasma treatment *J. Appl. Microbiol.* **109** 2039
19) Shivaji S, Chaturvedi P, Suresh K, Reddy G S, Dutt C B, Wainwright M, Narlikar J V and Bhargava P M 2006 *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes *Int. J. Syst. Evol. Microbiol.* **56** 1465
20) Cooper M, Fridman G, Staack D, Gutsol A F, Vasilets V N, Anandan S, Cho Y I, Fridman A and Tsapin A 2009 Decontamination of Surfaces From Extremophile Organisms Using Nonthermal Atmospheric-Pressure Plasmas *IEEE Trans. Plasma Sci.* **37** 866
21) Lunov O, Zablotskii V, Churpita O, Jäger A, Polívka L, Syková E, Dejneka A and Kubinová Š 2016 The interplay between biological and physical scenarios of bacterial death induced by non-thermal plasma *Biomaterials* **82** 71
22) Park Y-O, Lee C-M, Kim M-S, Jung S-C, Yang S-W, Kook M-S and Kim B-H 2016 In vitro bactericidal efficacy of atmospheric-pressure plasma jet on titanium-based implant infected with *Staphylococcus aureus Jpn. J. Appl. Phys.* **56** 01AC01
23) Pompl R *et al.* 2009 The effect of low-temperature plasma on bacteria as observed by repeated AFM imaging *New J. Phys.* **11** 115023
24) Yamaoka S, Kondo H, Hashizume H, Ishikawa K, Tanaka H and Hori M 2019 Plasma-activated solution alters the morphological dynamics of supported lipid bilayers observed by high-speed atomic force microscopy *Appl. Phys. Express* **12** 066001
25) Mendis D A, Rosenberg M and Azam F 2000 A note on the possible electrostatic disruption of bacteria *IEEE Trans. Plasma Sci.* **28** 1304
26) Kuwahata H, Yamaguchi T, Ohyama R and Ito A 2015 Inactivation of Escherichia coli using atmospheric-pressure plasma jet *Jpn J. Appl. Phys.* **54** 01AG08
27) Mai-Prochnow A, Clauson M, Hong J and Murphy A B 2016 Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma *Sci. Rep.* **6** 38610
28) Han L, Patil S, Boehm D, Milosavljević V, Cullen P J and Bourke P 2016 Mechanisms of Inactivation by High-Voltage Atmospheric Cold Plasma Differ for Escherichia coli and Staphylococcus aureus *Appl. Env. Microbiol.* **82** 450
29) Harold F M 1986 The vital force: a study of bioenergetics, WH Freeman, New York
30) Brun P, Bernabè G, Marchiori C, Scarpa M, Zuin M, Cavazzana R, Zaniol B and Martines E 2018 Antibacterial efficacy and mechanisms of action of low power atmospheric pressure cold plasma: membrane permeability, biofilm penetration and antimicrobial sensitization *J. Appl. Microbiol.* **125** 398
31) Martines E, *et al.* 2009 A novel plasma source for sterilization of living tissues *New J. Phys.* **11** 115014
32) Sharkey M A, Chebbi A, McDonnell K A, Staunton C and Dowling D P. 2015 Evaluation of the sensitivity of bacterial and yeast cells to cold atmospheric plasma jet treatments *Biointerphases* **10** 029507
33) Zimmermann J L, Shimizu T, Schmidt H-U, Li Y-F, Morfill G E and Isbary G 2012 Test for bacterial resistance build-up against plasma treatment *New J. Phys.* **14** 073037
34) Matthes R, Assadian O and Kramer A 2014 Repeated applications of cold atmospheric pressure plasma does not induce resistance in Staphylococcus aureus embedded in biofilms *GMS Hygiene Inf. Contr.* **9** Doc17
35) Mai-Prochnow A, Bradbury M, Ostrikov K and Murphy A B 2015 *Pseudomonas aeruginosa* Biofilm Response and

Resistance to Cold Atmospheric Pressure Plasma Is Linked to the Redox-Active Molecule Phenazine *PLoS ONE* **10** e0130373
36) Krewing M, Jarzina F, Dirks T, Schubert B, Benedikt J, Lackmann J-W and Bandow J E 2019 Plasma-sensitive *Escherichia coli* mutants reveal plasma resistance mechanisms *J. R. Soc. Interface* 20180846
37) Neumann E, Schaefer-Ridder M, Wang Y and Hofschneider P H 1982 Gene transfer into mouse lyoma cells by electroporation in high electric fields *EMBO J.* **1** 841
38) Felgner P L, Gadek T R, Holm M, Roman R, Chan H W, Wenz M, Northrop J P, Ringold G M and Danielsen M 1987 Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure *Proc. Natl Acad. Sci. USA* **84** 7413
39) Kwon I and Schaffer D V 2008 Designer gene delivery vectors: molecular engineering and evolution of adeno-associated viral vectors for enhanced gene transfer *Pharm. Res.* **25** 489
40) Ogawa Y, Morikawa N, Ohkubo-Suzuki A, Miyoshi S, Arakawa H, Kita Y and Nishimura S 2005 An epoch-making application of discharge plasma phenomenon to gene-transfer *Biotechnol. Bioeng.* **92** 865
41) Sakai Y, Khajoee V, Ogawa Y, Kusuhara K, Katayama Y and Hara T 2006 A novel transfection method for mammalian cells using gas plasma *J. Biotechnol.* **121** 299
42) Leduc Guay M D, Leask R L and Coulombe S 2009 Cell permeabilization using a non-thermal plasma *New J. Phys.* **11** 115021
43) Leduc M, Guay D, Couloube S and Leask R L 2010 Effects of non-thermal plasmas on DNA and mammalian cells *Plasma Process. Polym.* **7** 899
44) Ramachandran N, Jaroszeski M and Hoff A M, 2008 Molecular Delivery to Cells Facilitated by Corona Ion Deposition *IEEE Trans. Nanobioscience* **7** 233
45) Chalberg T W, Vankov A, Molnar F E, Butterwick A F, Huie P, Calos M P and Palanker D V 2006 Gene Transfer to Rabbit Retina with Electron Avalanche Transfection *Invest. Ophthalm. Visual Sci.* **47** 4083
46) Connolly R J, Lopez G A, Hoff A M and Jaroszeski M J 2009 Plasma facilitated delivery of DNA to skin *Biotechnol. Bioeng.* **104**, 1034
47) Connolly R J, Lopez G A, Hoff A M and Jaroszeski M J 2010 Characterization of plasma mediated molecular delivery to cells in vitro *Int. J. Pharm.* **389** 53
48) Nakajima T, Yasuda H, Kurita H, Takashima K and Mizuno A 2011 Generation of bactericidal factors in the liquid phase and approach to new gene transfer technology by low temperature plasma jet treatment *Int. J. Plasma Environ. Sci. Technol.* **5** 42
49) Edelblute C M, Heller L C, Malik M A and Heller R 2015 Activated air produced by shielded sliding discharge plasma mediates plasmid DNA delivery to mammalian cells *Biotechnol. Bioeng.* **112** 2583
50) Xu D, Wang B,Xu Y, Chen Z, Cui Q, Yang Y, Chen H and Kong M G 2016 Intracellular ROS mediates gas plasma-facilitated cellular transfection in 2D and 3D cultures *Sci. Rep.* **6** 27872
51) Sasaki S, Kanzaki M and Kaneko T 2014 Highly efficient and minimally invasive transfection using time-controlled irradiation of atmospheric-pressure plasma *Appl. Phys. Express* **7** 026202
52) Kaneko T, Sasaki S, Hokari Y, Horiuchi S, Honda R and Kanzaki M 2015 Improvement of cell membrane permeability using a cell-solution electrode for generating atmospheric-pressure plasma *Biointerphases* **10** 029521
53) Sasaki S, Honda R, Hokari Y, Takashima K, Kanzaki M and Kaneko T. 2016 Characterization of plasma-induced cell membrane permeabilization: focus on OH radical distribution *J. Phys. D: Appl. Phys.* **49** 334002
54) Kaneko T, Sasaki S, Takashima K and Kanzaki M 2017 Gas liquid interfacial plasmas producing reactive species for cell membrane permeabilization *J. Clin. Biochem. Nutr.* **60** 3
55) Sasaki S, Kanzaki M, Hokari Y, Tominami K, Mokudai T, Kanetaka H and Kaneko T 2016 Roles of charged particles and reactive species on cell membrane permeabilization induced by atmospheric-pressure plasma irradiation *Jpn. J. Appl. Phys.* **55** 07LG04
56) Sasaki S, Kanzaki M and Kaneko T 2016 Calcium influx through TRP channels induced by short-lived reactive species in plasma-irradiated solution *Sci. Rep.* **6** 25728
57) Ikeda Y, Motomura H, Kido Y, Satoh S and Jinno M 2016 Effects of molecular size and chemical factor on plasma gene transfection *Jpn. J. Appl. Phys.* **55** 07LG06
58) Jinno M, Tachibana K, Motomura H, Saeki N and Satoh S 2016 Improvement of efficiency and viability in plasma gene transfection by plasma minimization and optimization electrode configuration *Jpn. J. Appl. Phys.* **55** 07LG09
59) Jinno M, Ikeda Y, Motomura H, Kido Y and Satoh S 2016 Investigation of plasma induced electrical and chemical factors and their contribution processes to plasma gene transfection *Arch. Biochem. Biophys.* **605** 59
60) Jinno M, Ikeda Y, Motomura H, Isozaki Y, Kido Y and Satoh S 2017 Synergistic effect of electrical and chemical factors on endocytosis in micro-discharge plasma gene transfection *Plasma Sources Sci. Technol.* **26** 065016
61) Hiramatsu T, Hirashige H, Kido Y, Satoh S and Jinno M 2019 Importance of collision frequency in the molecular size dependency of gene transfer efficiency in the surface discharge method *Jpn. J. Appl. Phys.* **58** SEEG05
62) Vijayarangan V, Delalande A, Dozias S, Pouvesle J M, Pichon C and Robert E 2018 Cold Atmospheric Plasma Parameters Investigation for Efficient Drug Delivery in HeLa Cells *IEEE Trans. Rad. Plasma Med. Sci.* **2** 109
63) Yusupov M, Van der Paal J, Neyts E C and Bogaerts A 2017 Synergistic effect of electric field and lipid oxidation on the permeability of cell membranes *Biochim. Biophys. Acta Gen. Subj.* **1861** 839