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Foundations of plasmas for medical applications

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Abstract

Plasma medicine refers to the application of nonequilibrium plasmas at approximately body temperature, for therapeutic purposes. Nonequilibrium plasmas are weakly ionized gases which contain charged and neutral species and electric fields, and emit radiation, particularly in the visible and ultraviolet range. Medically-relevant cold atmospheric pressure plasma (CAP) sources and devices are usually dielectric barrier discharges and nonequilibrium atmospheric pressure plasma jets. Plasma diagnostic methods and modelling approaches are used to characterize the densities and fluxes of active plasma species and their interaction with surrounding matter. In addition to the direct application of plasma onto living tissue, the treatment of liquids like water or physiological saline by a CAP source is performed in order to study specific biological activities. A basic understanding of the interaction between plasma and liquids and bio-interfaces is essential to follow biological plasma effects. Charged species, metastable species, and other atomic and molecular reactive species first produced in the main plasma ignition are transported to the discharge afterglow to finally be exposed to the biological targets. Contact with these liquid-dominated bio-interfaces generates other secondary reactive oxygen and nitrogen species (ROS, RNS). Both ROS and RNS possess strong oxidative properties and can trigger redox-related signalling pathways in cells and tissue, leading to various impacts of therapeutic relevance. Dependent on the intensity of plasma exposure, redox balance in cells can be influenced in a way that oxidative eustress leads to stimulation of cellular processes or oxidative distress leads to cell death. Currently, clinical CAP application is realized mainly in wound healing. The use of plasma in cancer treatment (i.e. plasma oncology) is a currently emerging field of research. Future perspectives and challenges in plasma medicine are mainly directed towards the control and optimization of

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CAP devices, to broaden and establish its medical applications, and to open up new plasma-based therapies in medicine.

Keywords: plasma medicine, redox biology, low temperature plasma, reactive species, cancer, wound healing, cold atmospheric pressure plasma (CAP)

(Some figures may appear in colour only in the online journal)

1. Introduction

Nonequilibrium plasmas are weakly ionized gases which contain charged species (electrons, positively and negatively charged ionic species), neutral species (atomic and/or molecular radicals and nonradicals), and electric fields. These plasmas also emit radiation that spans wavelengths in the infrared, visible, and ultraviolet (UV) ranges. Generally, the degree of ionization in such plasmas is less than 0.001% and their gas temperatures are relatively low, typically less than 100 °C. These plasmas can be generated at low pressures as well as at atmospheric pressure and they are often referred to as cold or low-temperature plasma. Low pressure plasmas have been used for many decades in the fabrication of semiconductor components. Atmospheric pressure plasmas have typically been used in material surface processing applications such as making materials more hydrophilic or more hydrophobic. Since the mid 1990s, atmospheric-pressure low-temperature plasmas have been used in biological and medical applications, which are the main focus of this paper. In the literature, different nomenclature is used for this type of plasmas, such as low-temperature atmospheric-pressure plasma (LTP), nonthermal plasma (NTP), tissue tolerable plasma, and others. In this paper, we basically use the name and abbreviation cold atmospheric-pressure plasma (CAP), which is the most commonly used.

Investigations into the interaction of CAP with biological cells and tissues showed that the effects of LTP are mediated primarily by chemically reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1, 2]. Both ROS and RNS possess strong oxidative properties and can trigger signaling pathways in biological cells. Therefore, delivering controllable doses of these species to cells and tissues can lead to specific biological outcomes including the onset of apoptosis, enhancement or suppression of cell proliferation, modified cell migration, etc. The ability to modulate cell behavior locally has crucial implications in various CAP-based biomedical applications such as wound healing and cancer treatment [3–12]. Moreover, experiments on eukaryotic cells demonstrated that CAP can affect mammalian cells without causing thermal or other damage. For example, the ability of CAP to inactivate pathogens while stimulating healthy skin cells is the basic concept behind the use of CAP for wound healing.

The generation and transport of the reactive species generated by CAP sources proceed in several stages. Charged species, metastable species, and other atomic and molecular reactive species are first produced in the core plasma region. These species are transported to the discharge afterglow until finally coming into contact with the biological targets, such as cells or tissues. In the plasma afterglow, where air diffusion into the feed gas channel occurs, other secondary reactive species such as O, OH, O_3, O_2^-, NO, and NO_2 are generated. Figure 1 is an illustration of the processes described above [1]. Studies on the effects of plasma-generated reactive species on biological cells showed that, depending on the operating conditions (power, gas type and flow rate, exposure time, distance between plasma source and target, etc), different outcomes can be achieved. These include cell death (via regulated cell death like necrosis or apoptosis), cell detachment, change in cellular morphology and heterotrophic pathways, change in cell motility, cell proliferation, etc [3–12].

As shown in figure 1, the plasma-generated reactive species first encounter a gas–liquid interface where intermediate chemical species are produced. These species then get solvated in the liquid where other reaction by-products are generated before direct interaction with the biological cells. Therefore, the rate of solvation of the various species and their lifetimes in physiological liquid need to be taken into account. Table 1 shows Henry's law solubility constants H^{cp} for several species of relevance for water at 298.15 K, while table 2 shows the half-life of various species in physiological liquids. Note that the larger Henry's constant, the deeper in the liquid a specie can penetrate with the diffusion distance roughly scaling with the square root of the half-life. In addition, it is to be understood that Henry's law is used here as a first estimation only, since CAP is in a nonequilibrium state.

The biological effects of many of the above-listed reactive species are well known in cell biology. These species can interact with cell membranes, enter the cells and increase the intracellular ROS concentrations, which may lead to DNA strand breaks, mitochondria damage, and may compromise the integrity of other organelles and macromolecules (such as proteins). For example, the hydroxyl radical (OH) causes peroxidation of unsaturated fatty acids which are a major component of the lipids constituting the cell membrane. Another byproduct of plasma application is hydrogen peroxide (H_2O_2), which possesses strong oxidative properties that affect, via the peroxide ions, lipids, proteins, and DNA. Nitric oxide (NO) is another species that can be generated by plasma as it interacts with air. The NO molecule is known to have several biological effects, which include the regulation of immune-deficiencies, induction of phagocytosis, proliferation of keratinocytes, and regulation of collagen synthesis. In general, ROS and RNS can also trigger

cell signaling cascades, modulate cell functions, and, beyond certain concentration thresholds, can ultimately lead to cellular death pathways such as necrosis or apoptosis. Other CAP generated agents that may play biological roles are charged particles (electrons, negative and positive ions), photons, as well as large electric fields that may cause irreversible cellular electroporation, allowing large molecules to enter the cells (see section 4.1).

Since plasma produces copious amounts of the above-mentioned species, it was realized early on that CAP can be used for therapeutic purposes. Many seminal experiments showed that this was indeed the case, which ultimately led to the emergence and development of the field of plasma medicine.

The use of plasma for potential therapeutic purposes dates back to the 19th century and even earlier. However, these early attempts were not based on in-depth scientific studies. In fact, at that time knowledge of cell biology, redox biology, and biochemistry was at a rudimentary stage. In addition, the plasma state was more of a mystery and not understood at all. Note that the electron was discovered in 1897 by J. J. Thomson while the proton was first observed by E. Goldstein in 1886 and identified as part of an atom nucleus by E Rutherford in 1911. The definition of plasma itself was only proposed in 1928 by I Langmuir. Moreover, it is important to realize that for most of the 20th century low temperature plasmas that were created in the laboratory operated mostly at low pressure conditions. Plasmas created at atmospheric pressure were thermal plasmas such as the ones produced by arc discharges and plasma torches, which were not suitable for biomedical applications. However, low temperature plasmas generated at atmospheric pressure became prevalent by the late 1980s and CAP that can safely come in contact with biological cells and tissues were developed even later. So, science-based 'modern' plasma medicine as we understand it today only started in the mid-1990s when atmospheric-pressure, low-temperature plasma was applied to inactivate bacteria and when it was first realized that the reactive species generated by the plasma played a crucial role in the observed results [13, 14]. This groundbreaking work was followed by seminal studies on the effects of plasma on eukaryotic/mammalian cells when it was shown that low doses of CAP can cause cell detachment without causing necrosis and under some conditions apoptosis can be achieved [15, 16]. Following the early foundational works mentioned above, plasma medicine, as an emerging medical field, reached critically important milestones when the first clinical trials on wound healing and cancer treatment were conducted [7, 17].

Up until now, several CAP sources have been approved for medical and cosmetic use. In 2008 the US FDA approved the Rhytec Portrait® plasma jet for use in dermatology. Other plasma devices that are in use today for various medical applications include the Bovie J-Plasma® and the Canady Helios Cold Plasma and Hybrid Plasma™ Scalpel. In Europe, the medical device certification class IIa was given to the first CAP-devices kINPen® (Neoplas Med GmbH, Greifswald, Germany), and PlasmaDerm® (CINOGY GmbH, Duderstadt, Germany) in 2013, and later on to SteriPlas® (ADTEC, Hunslow, UK) and plasma care® (Terraplasma Medical, Garching, Germany) (see section 5 of this paper).

Table 1. Henry's law solubility constant H^{cp} for various species in water at 298.15 K (source: Jet Propulsion Laboratory data base).

Species	H^{cp} (mol/l × atm.)
Argon (Ar)	1.4×10^{-3}
Oxygen (O_2)	1.2×10^{-3}
Ozone (O_3)	1.1×10^{-2}
Hydroxyl (OH)	2.9×10^{1}
Hydrogen peroxide (H_2O_2)	8.4×10^{4}
Nitric oxide (NO)	1.9×10^{-3}
Nitrogen dioxide (NO_2)	4.0×10^{-2}

Table 2. Estimated half-life of various species in physiological environments. Note that the actual lifetime depends on factors such as pH and concentrations of reactive partners.

Specie	Estimated half-life
OH·	ns
NO_2·	μs
O_2^-	s
$ONOO^-$	ms
NO·	1–10 s
$^1O_2$	3–10 μs
H_2O_2	Minutes
NO_2^-	Minutes
NO_3^-	Minutes

In the last two decades the field has experienced exponential growth in term of the number of studies conducted worldwide, the number of research centers, institutes, laboratories, and groups active in the various aspects of the field, as well as in terms of the large number of peer reviewed publications. Finally, and as evidence that the field has steadily been reaching a level of maturity, several books covering the fundamentals as well as the applications of CAP in medicine were published within the last decade [18–22].

2. Plasma as a therapeutic instrument

2.1. Fundamentals of low temperature plasma

CAPs are weakly ionized gases where the electrons exhibit kinetic temperatures much higher than that of the ions and neutrals. Because of this particular nonequilibrium, the gas does not undergo extensive heating. One key design feature that characterizes CAP sources is the avoidance of the glow-to-arc transition. This can be achieved by various means including judicious electrodes design and/or wave-tailoring of the applied power. For example, barrier discharges use a dielectric or a resistive layer to cover one or both electrodes. This way the discharge current is self-limited, and the discharge is inhibited from turning into a spark. On the other hand, using fast rise time short pulses allows the energy to be exclusively coupled to the electron population and in this way, the gas temperature remains relatively low. Pulses shorter than the glow-to-arc transition time are an ideal solution to maintain the nonequilibrium characteristic of a discharge.

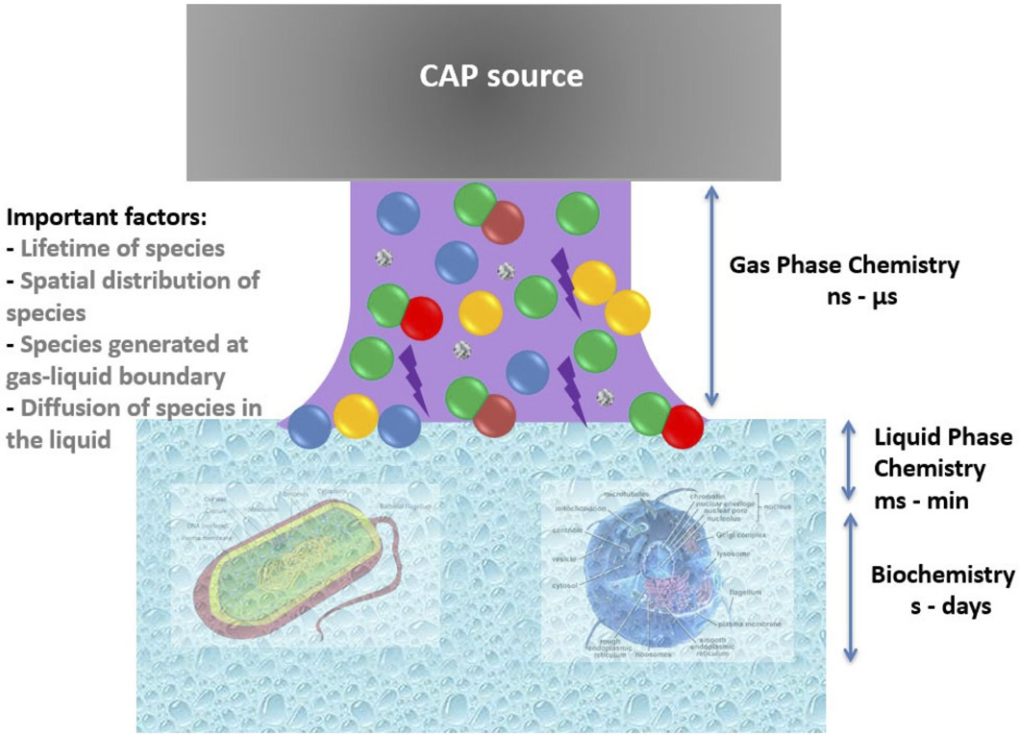

Figure 1. Schematic depiction of low temperature plasma (plasma jet) interacting with biological targets. Cells are typically covered by biological fluids, so the plasma first interacts with a liquid layer. Secondary and tertiary reaction by-products generated in the liquid then interact with the biological cells.

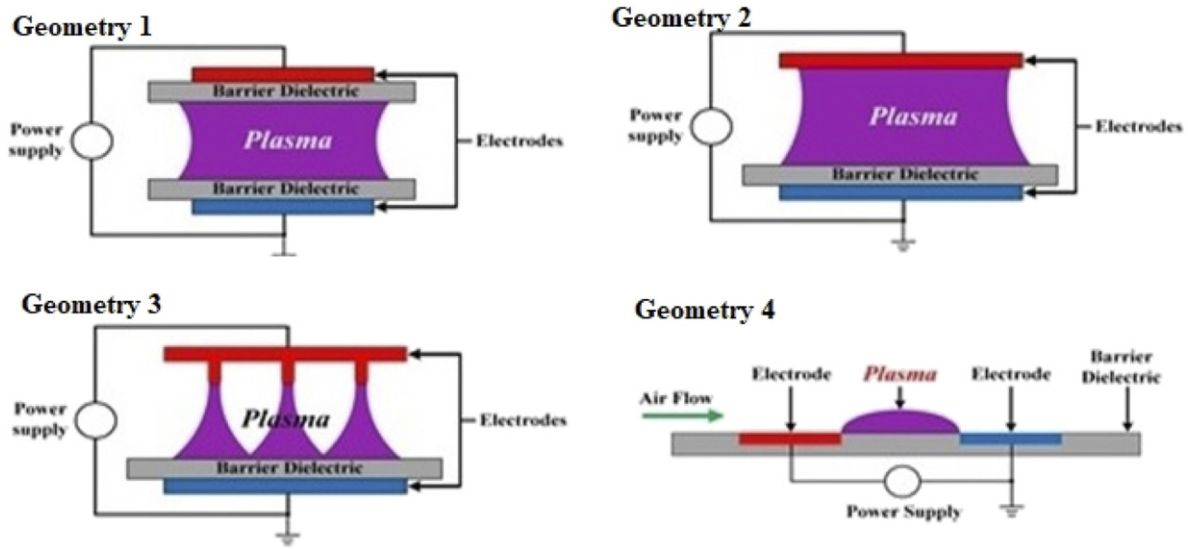

Figure 2. DBD with various configurations and electrode geometry.

Nonequilibrium, low temperature plasma offers a unique medium that can be used to interact with biotic matter to selectively induce certain biological outcomes. The effects of CAP on biological cells are mainly mediated by its ROS and RNS (see section 4.1) [1].

In plasma medicine two types of low temperature plasma sources have been extensively used, the dielectric barrier discharge (DBD) and the nonequilibrium atmospheric pressure plasma jets (N-APPJ). The following are brief descriptions of these two sources.

2.1.1. The dielectric barrier discharge. One of the earliest methods to generate CAPs is to cover planar or cylindrical electrodes by a dielectric material. The electrodes are separated by a small gap where the operating gas is introduced. The electrodes are driven by voltages of several kV at frequencies in the kHz range. This electrode scheme allowed the generation of stable discharges that can be filamentary or diffuse depending on parameters such as frequency, voltage amplitude and gas mixture. Common dielectric materials used in DBDs include glass, quartz, ceramics and polymers. The gap distance

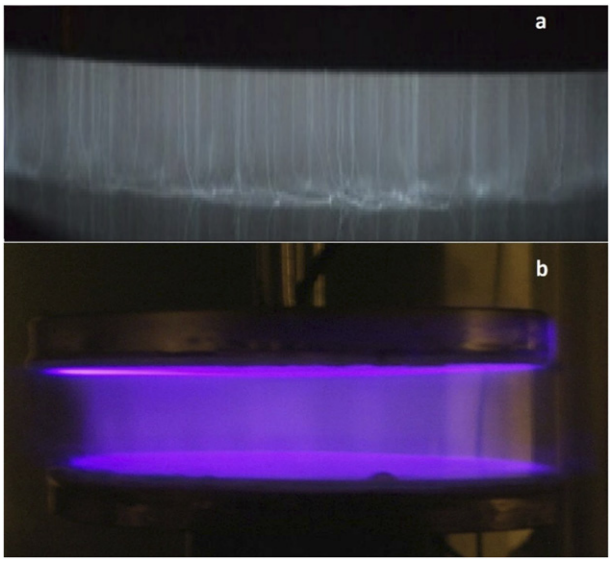

Figure 3. Images of a DBD-generated filamentary plasma (a) and that of diffuse DBD plasma (b). Images (a) and (b) are by Mounir Laroussi.

between electrodes varies from less than 0.1 mm in plasma displays, to several millimeters in ozone generators and up to several centimeters in CO_2 lasers. DBD devices can be made in many different geometries, including planar and cylindrical. Figure 2 shows the schematic of different types of DBD, including volumetric and surface discharges. Figure 3 shows images of a filamentary DBD and a diffuse DBD plasma.

The arrangement of electrodes is generally contained within a vessel to allow for the introduction of a defined gaseous mixture. When the discharge is ignited electric charges accumulate on the dielectric surface. These surface charges cause a drop in the voltage across the gas gap and a sharp decrease of the discharge current. In the filamentary regime, the discharge current exhibits multiple erratic current spikes per half cycle. However, when the discharge is diffuse the current exhibits only a few current peaks or 'humps' per half cycle being in phase with the high voltage. Massines *et al* proposed a Townsend-type breakdown or a glow discharge, depending on the gas used, supported by seed electrons as well as surface memory processes (secondary electron emission by metastable molecule impact) [23]. For more in-depth coverage of the DBD the reader can consult reviews [24–27].

The electron energy distribution function (EEDF) plays a crucial role in nonequilibrium discharges. It is through electron impact excitation and ionization that the charged particles, excited species, and radicals are produced. Therefore, by tailoring the EEDF the plasma chemistry can be controlled to a certain extent. One method to achieve such control of the EEDF was to use repetitive short high voltage pulses. These applied pulses allow for the transfer of power exclusively to the electron population. This translates to enhanced excitation and ionization. In addition, pulses with widths of less than the characteristic time of the onset of the glow-to-arc transition help keep the plasma stable and inhibits it from turning into a spark. Investigators reported that for pulsed DBDs two discharges occur for every applied voltage pulse [28]. The first discharge (or 'primary' discharge), was ignited at the rising edge of the applied voltage pulse while the second discharge (or 'secondary' discharge), was self-ignited during the falling edge of the applied voltage pulse.

2.1.2. Nonequilibrium atmospheric plasma jets. N-APPJs are devices that can emit low temperature plasma plumes in the surrounding air. These plasma sources can generate a stable thin column of plasma outside the confinement of electrodes and into the surrounding environment. Because the plasma propagates away from the high voltage region and into a region where there is no externally applied electric field the plasma is electrically safe in the sense that it does not cause electrical shock to the treated biological targets. However, the plasma plume may exhibit a very high instantaneous local electric field at its tip.

N-APPJs come in various designs and geometries [29]. Investigators have used DC, pulsed DC, RF, and microwave power to drive these plasma jets. Noble gases (such as helium and argon) and gas mixtures (such as He/air, Ar/air, He/O_2, Ar/O_2, etc) are typically made to flow through at gas flow rates in the 1–10 slm range. Figure 4 shows schematics of a few examples of N-APPJs while figure 5 shows photographs of four different plasma jets.

Investigators discovered that the plasma plumes emitted by N-APPJs are not continuous but are in fact discrete plasma segments that are known as 'plasma bullets'. These plasma bullets propagate at hypersonic velocities, up to 10^5 m s^{-1} [30, 31]. Extensive experimental and modeling studies resulted in a good understanding of the physical mechanisms behind the generation and propagation of the plasma bullets [32–43]. Lu and Laroussi first proposed a photoionization model to explain the dynamics of the plasma bullets [31]. Further studies showed that the plasma bullets are in fact ionization waves which are guided by the gas channel, hence the name 'guided ionization waves' [44]. The electrical field at the head of the ionization waves plays a crucial role in the propagation process and was experimentally measured by various investigators to be in the 10–30 kV cm^{-1} range [45–47]. Figure 6 shows a simulation of the electric field generated at the head of the plasma plume as a function of axial position and time. To learn more about N-APPJs the reader can consult reviews [29, 48–50].

Readers who are interested in learning the basic physical laws that govern low temperature plasmas can find a thorough coverage in the foundation paper [51]. This includes discharge ignition, gas breakdown, Paschen law, diffusion, scaling laws, sheath dynamic, streamer mechanisms, etc. In addition, the fundamental concepts behind atmospheric pressure nonequilibrium plasma, which are of great relevance to the plasma sources used in plasma medicine, can be found in the foundation paper [52]. These include scaling laws, timescales, discharge inception and breakdown mechanisms, plasma instabilities, stabilization methods, etc [52].

2.2. Plasma reactive species

DBDs and N-APPJs produce a 'cocktail' of chemically reactive species in the gaseous phase including ROS such

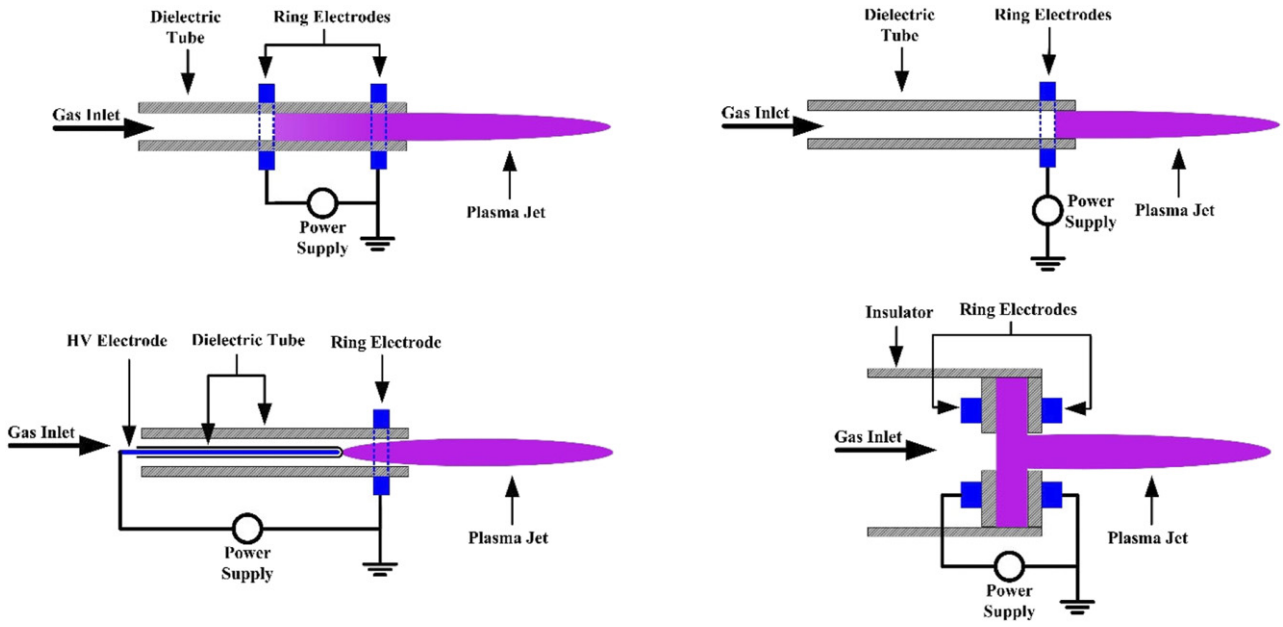

Figure 4. Schematics of four N-APPJs with different electrode configurations.

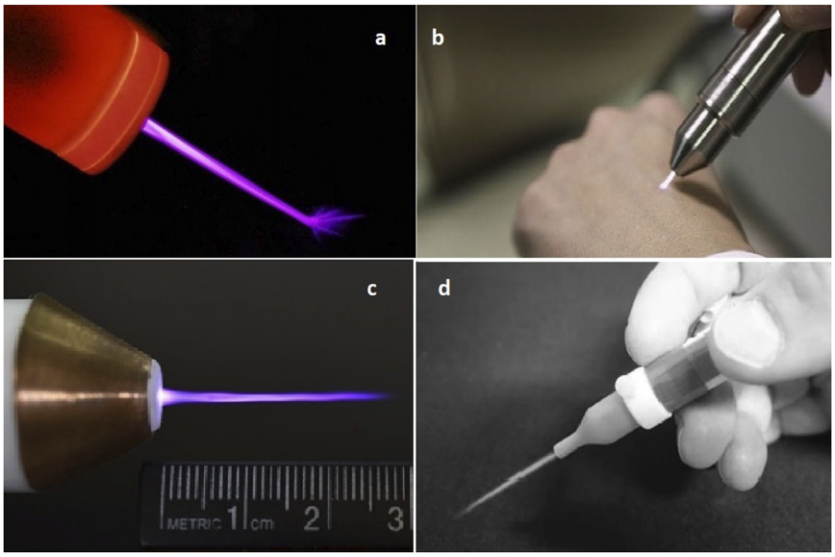

Figure 5. Photographs of N-APPJs. (a) The plasma pencil driven by pulsed DC (image by ODU/M. Laroussi); (b) the kINPen driven by RF power (image by INP/Th. von Woedtke); (c) Plasma jet driven by DC. Reproduced with permission from A. Shashurin; (d) Plasma jet driven by a piezoelectric transformer. Reproduced with permission from C. Tendero.

as hydroxyl, OH, atomic oxygen, O, singlet delta oxygen, $O_2(^1\Delta)$, superoxide, O_2^-, ozone, O_3, and hydrogen peroxide, H_2O_2. RNS such as nitric oxide, NO, and nitrogen dioxide, NO_2, are also generated. When interacting with liquids other secondary and tertiary species such as nitrite, NO_2^-, nitrate, NO_3^-, peroxynitrite, $ONOO^-$, peroxynitrous acid, ONOOH, organic radicals, RO, are also generated. Detailed discussion of the reactive species generated in liquids under CAP exposure will be presented in section 3 of this paper. As mentioned earlier, the concentrations and fluxes of these species play crucial roles when CAP interacts with biological matter. Therefore, determining these concentrations qualitatively and quantitatively is of paramount importance. The following provide some examples of these measurements and briefly describe the diagnostics methods used to obtain them.

As illustrative examples of CAP-produced reactive species, figure 7 through figure 9 show two important species generated by plasma jets. Figure 7 shows the radial distribution of hydroxyl, OH, for different gas flow rates while figure 8 shows the OH distribution in the presence of a target material. Figure 7 illustrates the major influence of the gas flow rate and indicates that the maximum OH density is close to the center of the plasma plume and decays with radial distance. As can be seen in figure 8, the presence of a target influences greatly the production of OH. This highlights the fact that the type of target has a dramatic influence on the OH concentration and its radial distribution, with the maximum density at the center followed by nearly exponential radial decay. A target with a dry surface leads to lower OH production while a wet surface results in a substantial increase in OH density. However, it is

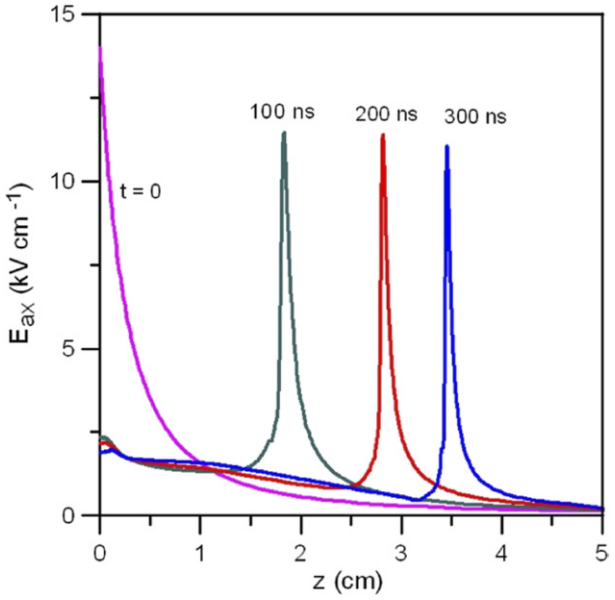

Figure 6. Profile of the electric field at the head of the plasma plume (outside of the plasma core area) of a pulsed plasma jet at various times and axial positions. Reproduced from [37]. © IOP Publishing Ltd. All rights reserved.

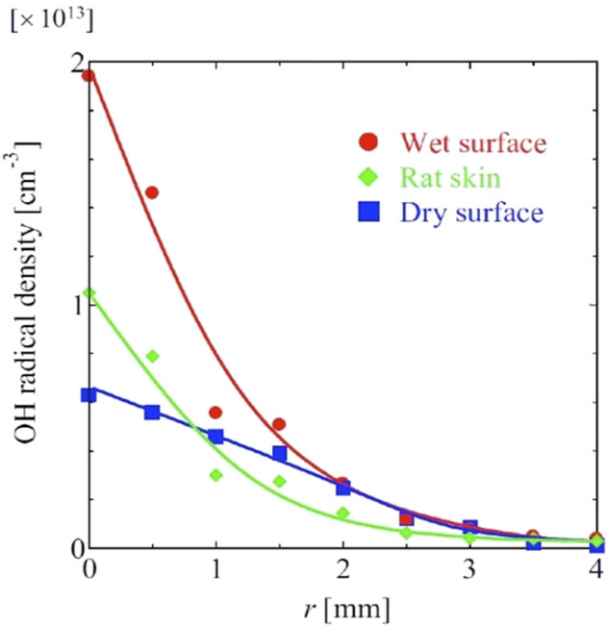

Figure 8. OH density radial distribution on wet/dry/rat skin surfaces measured using LIF. Reproduced from [54]. © IOP Publishing Ltd. All rights reserved.

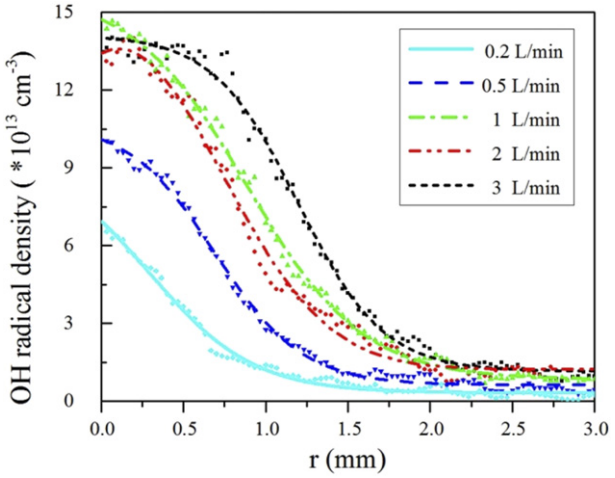

Figure 7. Radial distribution of the OH density for various gas flow rates measured using laser-induced fluorescence (LIF) (gas: helium with 115 ppm of H_2O). The plasma jet has an insulated single pin electrode inside a quartz tube and the plasma plume is applied on top of a water film. © [2016] IEEE. Reprinted, with permission, from [53].

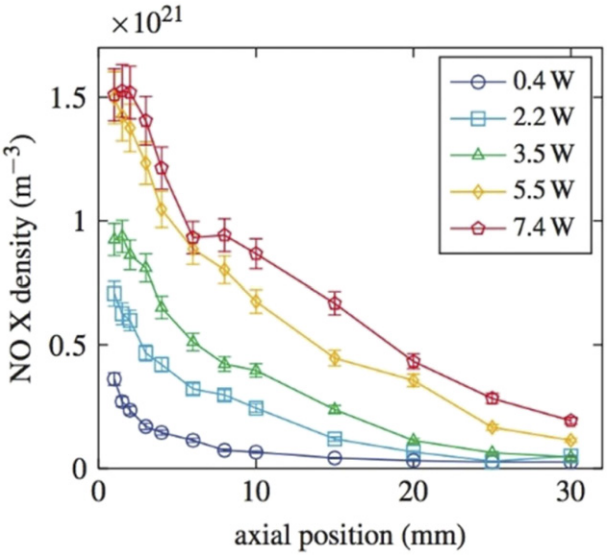

Figure 9. Axial profiles of NO concentration for different RF discharge power measured using LIF. The plasma is operated at a frequency of 13.6 MHz, pulsed at 20 kHz with 20% duty cycle. Working gas is Ar at 1 L min^{-1} with 2% air admixture. Reproduced from [55]. © IOP Publishing Ltd. All rights reserved.

important to keep in mind that the concentrations of species produced by plasma jets also depend on several other factors including the humidity level of the surrounding environment, gas flow rate, air mole fraction, etc. Figure 9 shows the axial distribution of nitric oxide, NO, generated by an RF jet at different applied powers, NO being a very important biological molecule. Figure 9 shows that the production of NO is a function of the power applied to the plasma and that its density decays rapidly with the axial distance away from the nozzle of the device.

Finally, figure 10 shows the concentrations of various species of biological relevance produced in the plasma plume of an argon N-APPJ source as a function of the axial position. As can be observed in the figure, some important species such as O, NO, NO_2, O_2^-, O_3, OH, H_2O_2, and HNO_2 remain at relatively high concentrations in the far effluent, at up to a few centimeters from the nozzle. Other species, however, are more prevalent within a short distance from the nozzle and decay to markedly low levels in the far effluent. Knowing these distributions helps inform the user which species are most likely to play a role and at which locations.

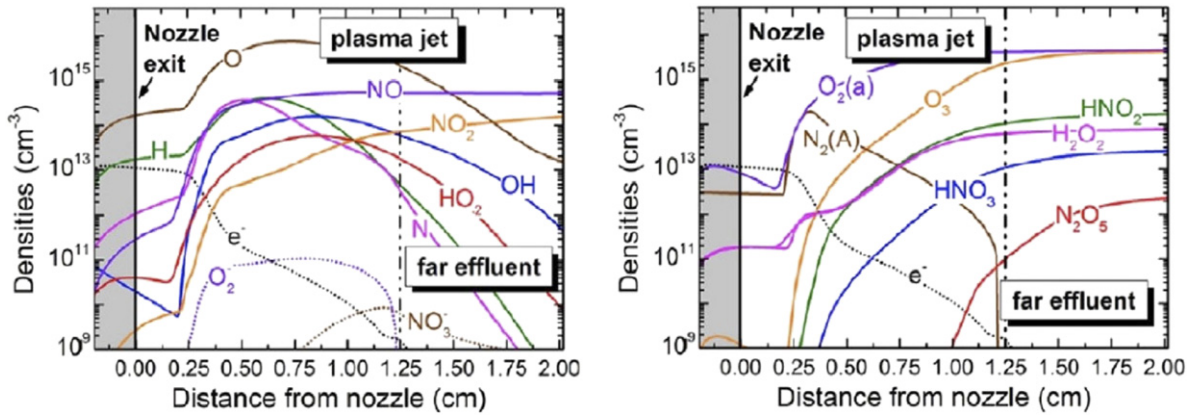

Figure 10. Axial concentrations of biomedically active species in an Ar plasma jet. Results are obtained from modeling. Reproduced from [56]. © IOP Publishing Ltd. All rights reserved.

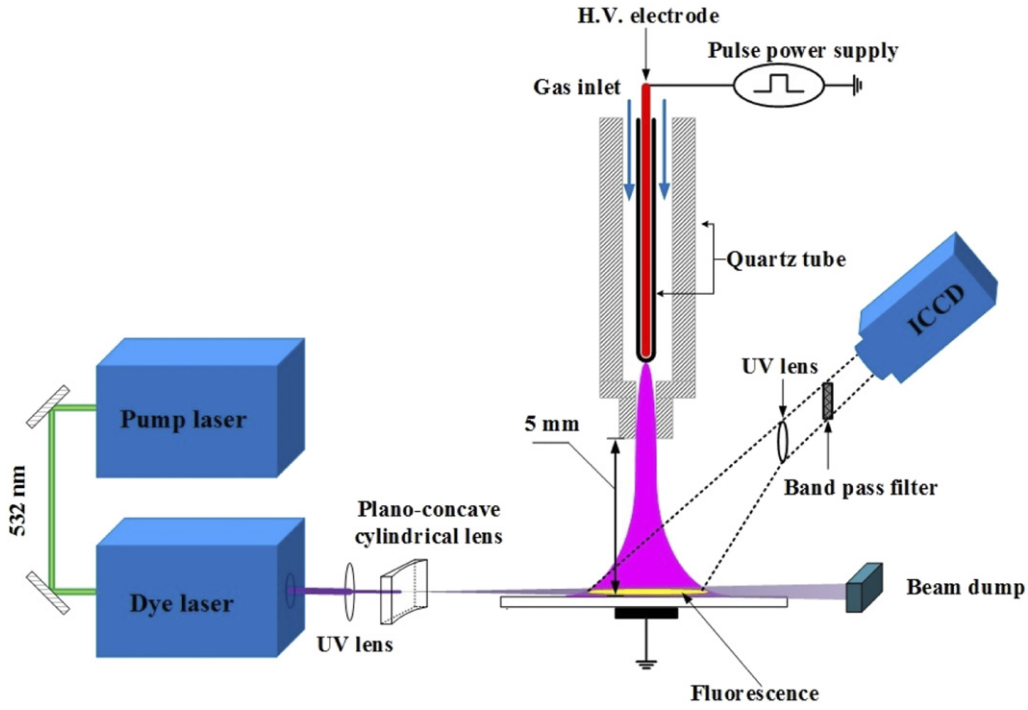

Figure 11. LIF setup to measure the concentration of OH. © [2016] IEEE. Reprinted, with permission, from [53].

To conclude this section, it is important to reiterate the fact that quantitative evaluation of the type of species, their concentrations and their axial and radial distributions is crucial for biomedical applications since the biological outcomes correlate directly with the fluxes of such species. To maximize a flux of certain species could lead to distress and cell death while a low flux of the same species could lead to eustress and cell proliferation (see section 4 of this paper).

2.3. Diagnostics methods for low temperature plasmas

In addition to well-established electrical characterization (to measure current, voltage, power) there are several diagnostics methods to identify and measure the concentrations of reactive species produced by low temperature plasmas. Because typically CAP sources have relatively small dimensions and exhibit nonuniformities, invasive techniques (such as electrical probes) are usually not suitable. Optical diagnostics are therefore preferred since they are mostly noninvasive and can yield measurements that are resolved in time and in space. These diagnostics techniques include optical emission spectroscopy (OES), optical absorption spectroscopy, laser induced fluorescence (LIF), and two photon absorption laser induced fluorescence (TALIF). In addition, scattering techniques such as Thomson, Rayleigh, and Raman scattering can be used. Other advanced spectroscopic methods such as cavity ring down spectroscopy have also been used to evaluate very low concentrations of some species [57]. Finally, well-established chemical analysis techniques including mass spectrometry and electron paramagnetic resonance (EPR) spectroscopy are at the investigators' disposal. In the following section, only a brief

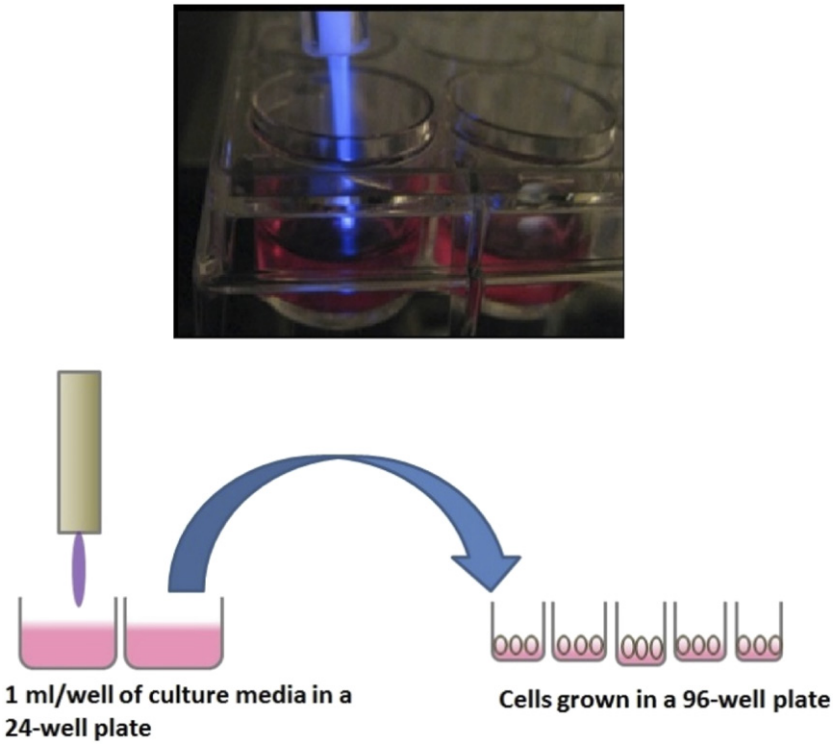

Figure 12. Indirect treatment: first, a liquid solution is exposed to LTP (top photograph), then the treated solution is applied on top of the cell (bottom schematic).

Table 3. Fraction of important bond dissociations and associated standard deviations upon impact of O, O_3, OH, and H_2O_2[a]. Reproduced with permission from [62], Copyright (2013) American Chemical Society.

Incident plasma species	C–N bond breaking events (%)	Ether C–O bond breaking events (%)	C–C bond breaking events (%)
O atoms	26 ± 6	78 ± 6	38 ± 7
O_3 molecules	8 ± 4	56 ± 7	26 ± 6
OH radicals	8 ± 4	54 ± 7	14 ± 5
H_2O_2 molecules	0	44 ± 7	12 ± 5

[a]Note that the values are calculated from 50 independent simulations for each incident species.

coverage of OES, LIF, and TALIF diagnostic techniques is presented. For more in-depth information on optical diagnostics methods the reader can consult the review papers [58, 59].

OES is a relatively simple technique that can yield both qualitative and quantitative measurements. OES is used to identify excited species and absolute OES can measure their absolute densities but requires careful calibration. Only excited species that emit radiation can be identified and/or measured by OES. Species in the ground state cannot be detected by OES, so they require other measurements methods such as LIF and TALIF. OES has been used to measure the gas temperature, electrons density, and electric field strength. The gas temperature is deduced from the rotational temperature in the case of molecular gases. For example, the rotational structure of the 0–0 band of the second positive system of nitrogen is often used to estimate the gas temperature. This is done by comparing experimental data with simulation data. The electron density can be found using the Stark broadening by exploring the Balmer transition H_β of hydrogen at 486.132 nm. However, this broadening is usually resolved only when the electron density is greater than 10^{13} cm^{-3}. OES can be used to measure the electric field strength by applying the polarization-dependent Stark splitting and shifting of the helium 447.1 nm line and its forbidden counterpart. To learn the details of the above mentioned OES techniques the reader can consult reference [59].

LIF allows us to measure species in the ground state. LIF is based on a two-step process. First, a photon is absorbed by an atom or molecule. As a result, the absorbing particle is excited to a higher energy state. The excited species then undergoes spontaneous radiative decay to a lower energy level and in the process releasing a photon at a different wavelength than that of the incident laser light. The emitted photons constitute the fluorescent light signal, which can be detected, and its intensity measured. Figure 11 shows an example of an LIF setup to measure the concentration of OH produced by a plasma jet. By calibration or via signal fitting process the concentration of the species can be deduced. LIF can be used to measure atomic or

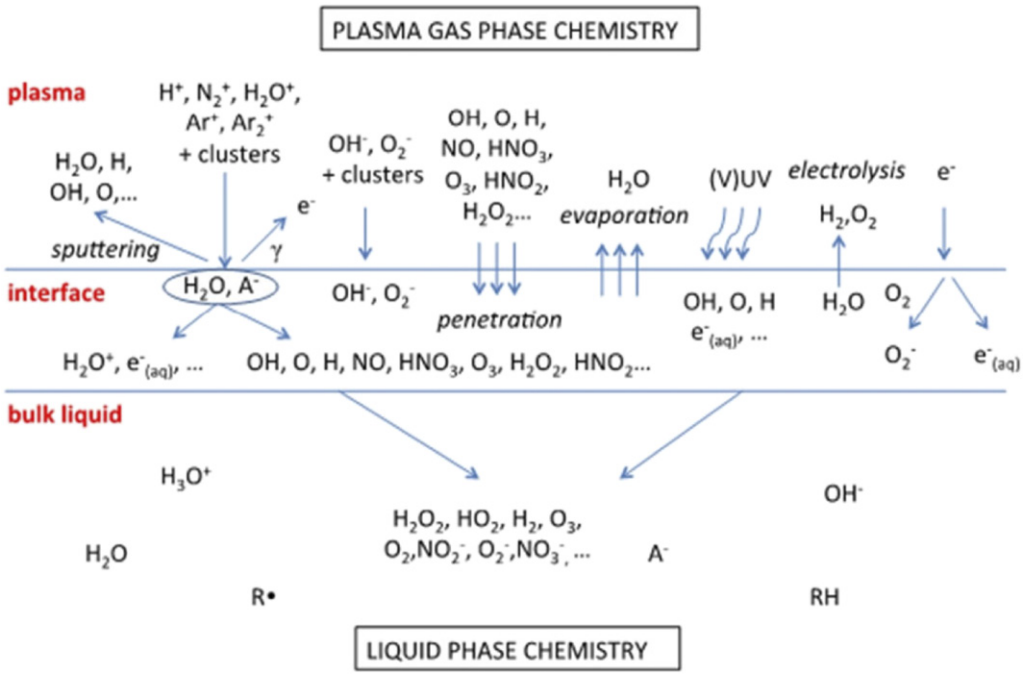

Figure 13. Main processes involved in the plasma–liquid interaction. The species represent the case of an Ar/air plasma interacting with water. Reproduced from [69]

molecular species, however, it is more complex for molecules. This is because vibrational and rotational energy transfers can influence the radiative decay. Reference [60] presents a good example of how LIF can be applied for nonequilibrium plasma.

In TALIF the probed species is first excited by a photon to an intermediate state. The intermediate level can be a virtual state or a real electronic state. A second photon excites the species into a final higher state and then after radiation decay to a lower state a fluorescence signal is emitted. As an example, TALIF has been used to measure the concentrations of atomic oxygen. In this case, a laser beam at a wavelength of 225.62 nm is used to excite atomic oxygen from the $2p^4\ ^3P^2$ state to the $3p^3\ ^3P_2$ state. The fluorescence signal is captured at a wavelength of 845 nm, which corresponds to the transition from $3p^3\ ^3P_2$ state to the $2s^3$ S state of O. The absolute concentration can be estimated by calibration methods. For more details, the reader can consult reference [58].

The above section discussed only measurements methods to evaluate concentrations of relevant species in the gaseous phase, generated by plasma. Section 3 of this paper discusses the methods used to measure concentrations of species generated in the liquid phase. There are well-established techniques, molecular probes, and assays to carry out such measurements**.** These include spectrophotometric assays (such as Griess assay for measuring nitrate), cholorimetric/fluorometric assays (such as Amplex red assay for measuring hydrogen peroxide), singlet delta oxygen sensor, chemical titration (for ozone), etc.

2.4. Methods of treatment

In plasma medicine the biological targets (cells, tissues, organs, etc) can be exposed to CAP in a direct or indirect way. In the case of sterilization/decontamination, the plasma can either come in direct contact with the cells (bacteria, viruses, fungi, biofilm, etc) or the cells can be placed in a location where they are exposed only to the plasma effluent or, in specific cases, to plasma processed air (indirect treatment). In direct treatment all the possible plasma-produced agents act on the cells. These include charged particles, reactive species, photons, electric field, and heat. In indirect treatment the contribution of charged and electronically excited particles, electric field, heat, and photons are greatly reduced or even eliminated. In this case, mostly the long-lived species generated by the plasma reach the biological target.

In the case of CAP treatment intended for medical therapies such as wound healing or cancer treatment, direct and indirect treatments take different meanings. Direct treatment can mean that the plasma or the plasma afterglow touches the target. Indirect treatment often means that CAP is first used to treat a liquid solution and then the plasma-treated solution is applied on top of cells, wounds, or injected into tumors. The used solutions include saline solution, cell culture media, ringer's lactate solution, etc. In this treatment modality the effects of heat, photons, and electric field are eliminated. In addition, under specific conditions plasma-treated solutions (PTS) can be stored and used at a later time (h day^{-1}). Figure 12 illustrates the steps involved in such treatment modality.

2.5. Modeling of CAP-cell and CAP-tissue interactions

This section briefly describes the computer models that have been used to simulate the interaction of CAP with biological matter. For extensive descriptions of the computer simulations

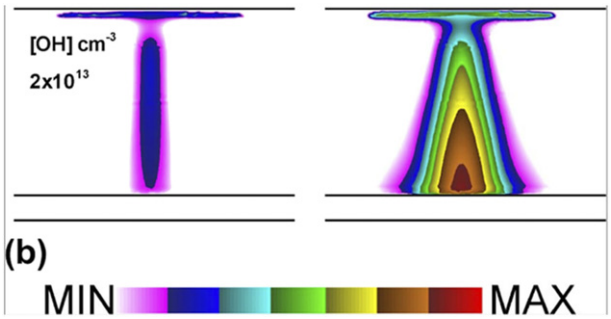

Figure 14. Effect of liquid evaporation on the gas-phase concentration of hydroxyl radicals. Reproduced from [72]. © IOP Publishing Ltd. All rights reserved.

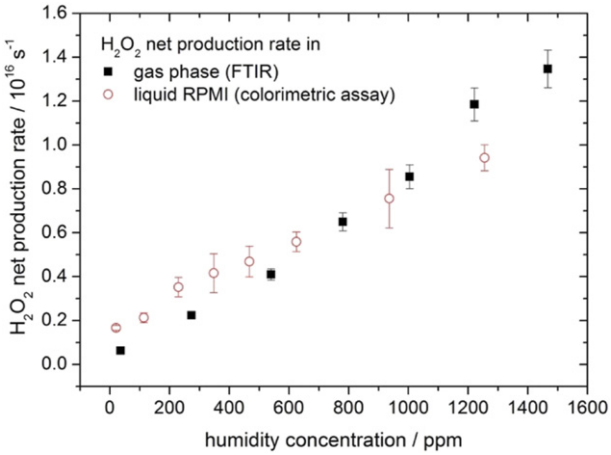

Figure 15. Gas-phase and liquid-phase concentration of hydrogen peroxide for different humidity concentrations. Reproduced from [73]. © IOP Publishing Ltd. All rights reserved.

used to model low temperature plasmas, the reader is referred the foundation paper by Alves *et al* [61].

A few researchers have attempted to model the interactions of low temperature plasma with biological cells and tissues. So far, two types of simulations approaches have been used, molecular dynamics (MD) simulations and hydrodynamic simulations. MD simulation (both classical MD and *ab initio* MD) is a method that allows the system to be described on the atomic level while hydrodynamic simulation is used on a more macroscopic level to describe the system on the cellular/tissue level. In the latter simulation method, the cells and tissues are modeled by dielectric materials having certain permittivity and conductivity. Using the above-mentioned methods, simulations of plasma interactions with bacteria, with eukaryotic/mammalian cells, and with tissues have been carried out [62, 63]. For example, MD simulations have been used to study the plasma effects on DNA, peptidoglycan, lipid bilayer, ion transport, and electroporation. These simulation studies are very important as they can shed light on phenomena on the atomic, molecular, subcellular, and cellular levels and can yield useful data that are hard or even impossible to obtain experimentally. The following are a few examples of what has been uncovered using such computer simulations.

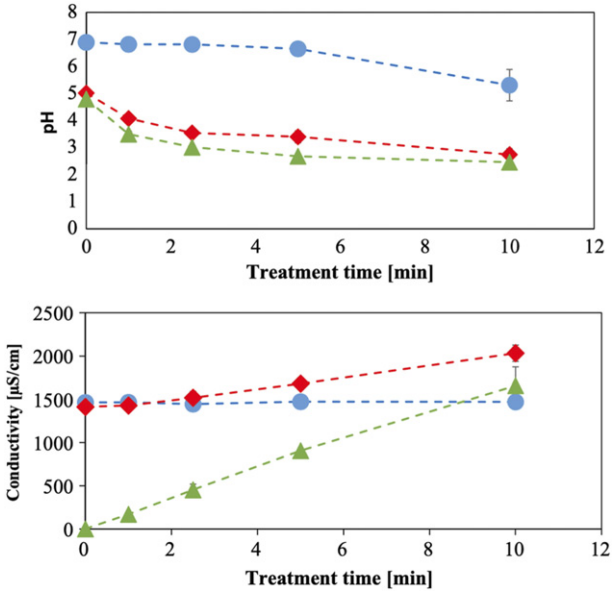

Figure 16. pH and conductivity variation during the air plasma treatment of DIW (green triangles), electrolyte solution (red diamonds) and phosphate buffered solution (blue circles). Reprinted from [74], Copyright (2015), with permission from Elsevier.

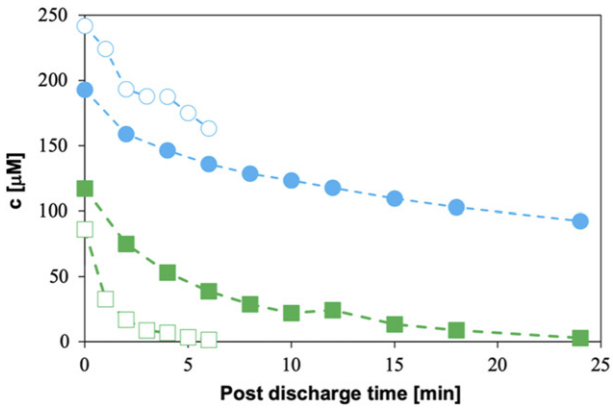

Figure 17. Evolution of the concentration of reactive species in DIW after air plasma treatments of 5 or 10 min (filled and empty symbols, respectively); green squares refer to H_2O_2 and blue circles to NO_2^-, the conjugate base of nitrous acid. Reprinted from [74], Copyright (2015), with permission from Elsevier.

To elucidate the effects of CAP on bacteria on fundamental and molecular levels, Yusupov *et al* conducted computer simulations on the interaction of ROS with bacterial peptidoglycan, which is a major constituent of the bacteria cell wall [62]. For this, they used reactive MD simulations and they found that ROS affect the structure of peptidoglycan by breaking the C–N, C–O, and C–C bonds. Table 3 shows the percentage of breaking events of the C–N, C–O, and C–C bonds and which plasma species is responsible for the breakage [62]. Note that, for example, hydrogen peroxide, H_2O_2, was found to be responsible for breaking the C–O and C–C bonds, which indicate that H_2O_2 specifically affects the disaccharide part of

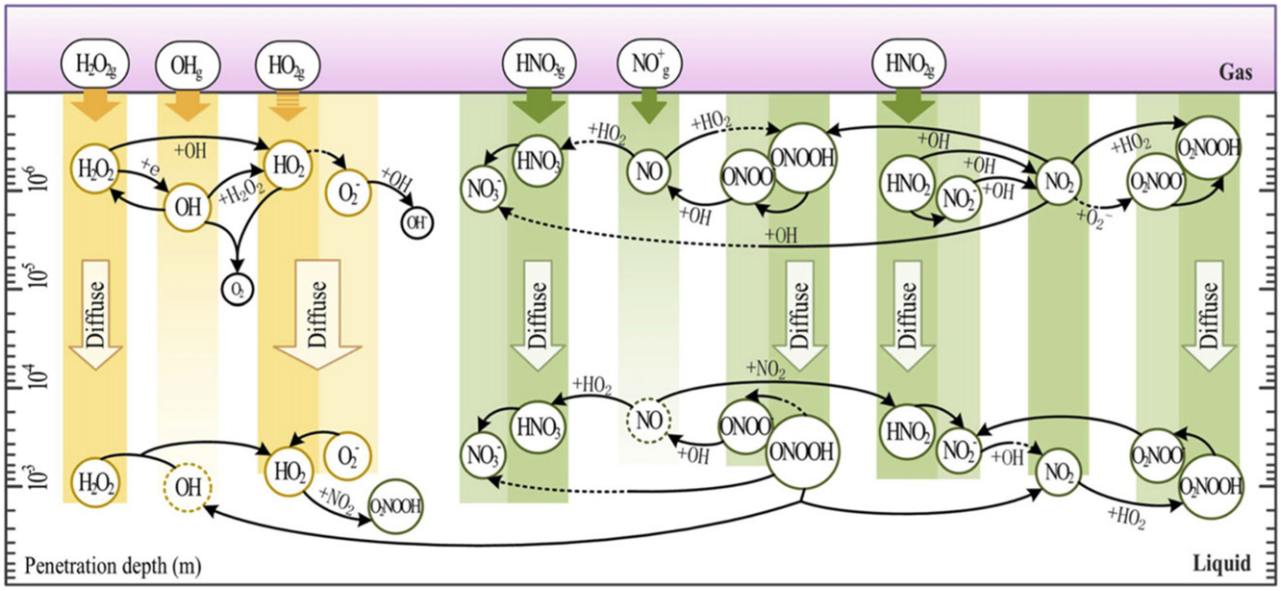

Figure 18. Schematic representation of the most important diffusion processes and chemical reactions taking place at the interface and in the bulk of plasma treated DIW. Reproduced from [82]. CC BY 4.0.

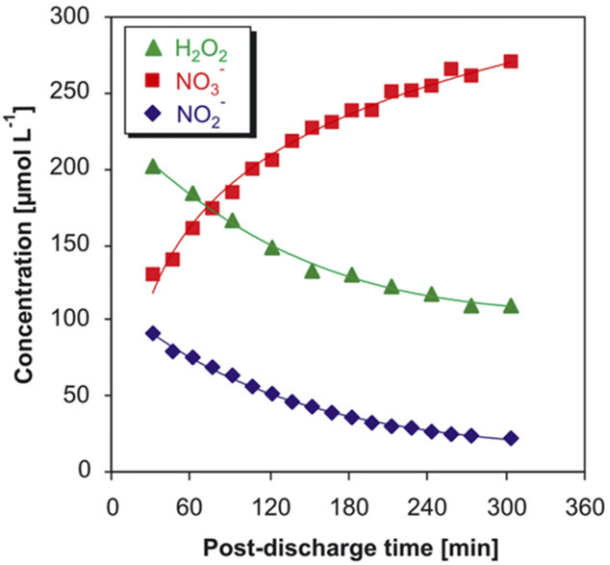

Figure 19. Evolution of the liquid phase concentration of H_2O_2, NO_2^- and NO_3^- after plasma treatment (pH 3.3). Reproduced from [84]. © IOP Publishing Ltd. All rights reserved.

the peptidoglycan. For a detailed description of the computational method used and all the simulation results, the reader is referred to reference [62].

Chen *et al* developed a molecular simulation of plasma chemistry that can link to functions on the cell and tissue levels [64]. In the case of biofilms, a reactive penetration model was used for mass transfer of transient plasma reactive species across the gas–liquid boundary. In the case of tissue, they used a fluid model and equations of heat and electric field transfer through the skin. Their results revealed that the penetration of plasma chemistry into hydrated biofilms is 10–20 μm deep, which correlated with the penetration of liquid-phase plasma chemistry dominated by ROS. With their model, they were also able to assess the thermal and electrical safety of CAP devices used on living tissue [64].

MD simulations have been used to study the electropermeabilization of cell membranes, ion transport through electropores, reactive species interaction with cell membranes and with biomolecular structures, lipid peroxidation, and interaction of reactive species with liquids. Detailed coverage of the computational methods and the results obtained in relation to these interactions can be found in reference [63].

Babaeva and Kushner carried out hydrodynamic simulations to study the propagation of electric field, plasma filaments through tissue, and fluxes of reactive species on tissues [65–67]. They used a 2D plasma hydrodynamic nonPDPSIM to study the propagation of streamers, the production of reactive and charged species as well as their fluxes on tissues and wounds. Using this model, they also investigated electroporation and the propagation of electric fields through skin and wounds. They found that the curvature of the skin influences greatly the propagation of the plasma filaments. As the filaments reach the skin surface, charge accumulation occurs resulting in the production of a lateral electric field that spreads the filaments over the surface of the wound. In addition, significant electric field penetration into the intracellular structure can occur to establish a high enough field for electroporation to occur. These investigators also quantified the fluxes of ROS and RNS, ions and photons towards the wound. They found that for typical DBD operating conditions, both the ROS and RNS show fluences over a treatment time of a few seconds comparable to those on the surface [65–67]. The same investigators also reported on the interaction of ROS and

RNS with liquid-covered wounds and showed that alkane-like hydrocarbons in the liquid influences which species reach the wound.

3. Plasma-induced effects in liquids and at bio-interfaces

The biological effects induced by plasma treatment revolve around the interaction of plasmas with a liquid. This aspect is almost obvious if we consider the case in which plasma is used to treat a liquid, which is then applied to exert a certain biological effect (indirect treatment). Nonetheless, even when the biological target is directly exposed to the plasma (direct treatment), liquids act as mediator of plasma effects since, under physiological conditions, a liquid environment surrounds cells and tissues. This liquid layer is typically a few hundred micrometers thick and its composition depends on the specific cells/tissue considered; as an example, the composition of the liquid layer in early-stage wounds is composed of 93% water and 7% proteins [68].

The main processes involved in the plasma–liquid interaction are shown in figure 13, where the reported species are those typically encountered in the case of an Ar/air plasma interacting with water. The schematic highlights the existence of an interface separating the plasma and bulk liquid region, where a number of key processes take place.

Over the course of the treatment, plasma components such as electrons, photons, radicals and gas-phase ROS and RNS penetrate the interface, diffusing and triggering chemical reactions with the formation of liquid-phase chemical species. These species can then diffuse out of the interface and reach the bulk liquid, where further chemical reactions take place. Liquid evaporation and sputtering at the interface add another layer of complexity, since these processes alter the gas-phase composition and, as a consequence, the type and concentration of plasma components flowing towards the liquid. The feedback effect of the liquid on the gas-phase chemistry is highlighted in figure 14, where modeling results for the case of an air plasma show that the gas-phase concentration of hydroxyl radicals (OH) is severely enhanced when evaporation takes place [70]. The interdependence of gas-phase chemistry and liquid-phase chemistry is further highlighted in figure 15, focusing on hydrogen peroxide (H_2O_2). The reported experimental results demonstrate that the humidity concentration influences the concentration of gas-phase H_2O_2, which in turn influences the liquid-phase concentration of H_2O_2 [71].

The liquid-phase chemistry certainly depends on the type and concentrations of the gas-phase species penetrating the interface, but also on the characteristics of the liquid itself. As an example, the Henry's constant (see table 1), indicating the liquid-phase concentration of a species having a certain partial pressure in gas-phase, is a function of both the diffusing species and the liquid. Also, liquid composition, conductivity and pH strongly affect the chemical reactions taking place in the aqueous phase. Interestingly, depending on the composition of the liquid, its conductivity and pH may vary over the course of the treatment, as shown in figure 16 for the case of deionized water (DIW), a phosphate buffered solution and an electrolyte solution [74].

Referring to the liquid-phase chemical species, a distinction i made between short-living and long-living species; the first term is used to refer to highly reactive species with a short lifetime, mainly involved in chemical reactions at the interface, while the second term refers to more stable species, populating the bulk of the liquid. In the field of plasma medicine, this distinction is often made on practical terms. Long-living species refer to those having a lifetime long enough to survive in the liquid for a certain amount of time after the plasma treatment, enabling indirect treatments. H_2O_2 and nitrous acid, HNO_2, are among the long living species always encountered after plasma treatment of aqueous solutions in the presence of air and humidity in the gas phase, as shown in figure 17 [74]. Short-living species, on the opposite, should play a role only in direct treatments. Although very practical, these definitions fail to account that many long-living species, over their lifetime, undergo chemical reactions, locally releasing highly reactive short-living species that can thus exert biological effects also in the case of indirect treatments.

The apparently straightforward figure 17 evidences the complexity of the liquid chemistry initiated by the plasma treatment and the interdependence of many factors. As an example, the fact that longer treatment times induce higher concentrations of H_2O_2 and nitrates (NO_2^-) in DIW, but also their faster degradation once the plasma treatment is over, can only be explained by a concurrent reduction of the solution pH, induced by the treatment itself and influencing the various reaction taking place in the liquid during and after the exposure to the discharge.

The aim of this chapter is to introduce the reader to the processes taking place in the gas–liquid interface and in the liquid phase. After describing the chemistry initiated by plasma in pure water, the case of liquids of more complex composition will be considered and techniques for the study of the effects induced by plasma in liquids will be introduced.

3.1. Plasma-induced chemistry in deionized water

This paragraph deals with the general aspects of plasma-induced liquid chemistry and with the reactions taking place in pure water, namely water without any organic or inorganic impurities. Experimentally, DIW is often used as a representative system for pure water, but a few clarifications are needed. As the term suggests, in DIW ions have been removed, resulting in a highly pure water with negligible conductivity and, at least immediately after its production, neutral pH. DIW exposure to air leads to the absorption of oxygen (O_2) and carbon dioxide, CO_2; the latter is responsible for the formation of carbonic acid, H_2CO_3, and the reduction of DIW pH below 7. These processes are worth mentioning since in most plasma-medical applications, the liquid is exposed to air before the plasma treatment. Another difference of DIW compared to distilled water is that the chemical process for its production does not alter uncharged and organic contaminants. It should be noted that, even if not perfectly representative of pure water,

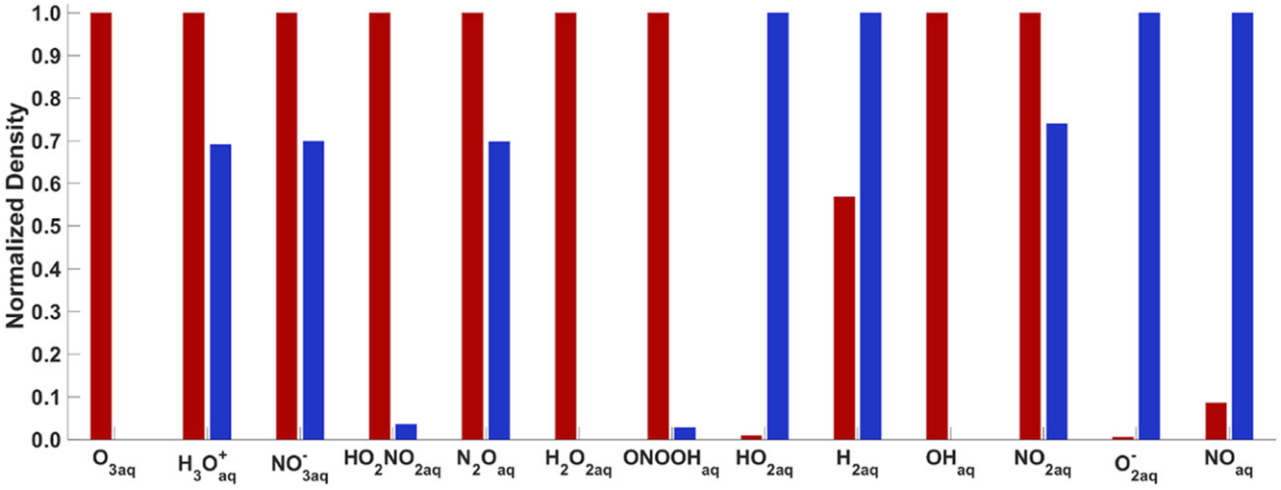

Figure 20. Concentration of chemical species in plasma-treated DIW with (blue) and without (red) biomolecules in the liquid. Reproduced from [83].

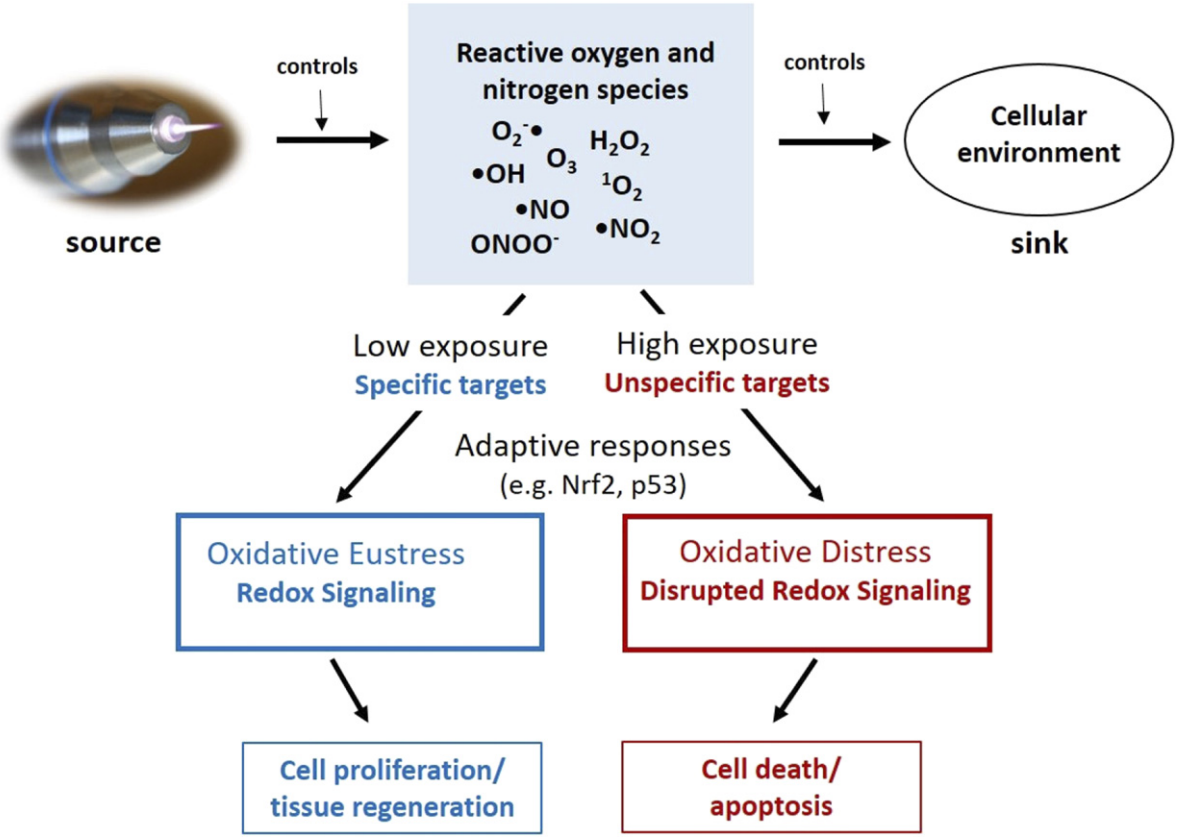

Figure 21. Schematic of the concept of plasma medicine as applied redox biology following the concept of oxidative eustress and distress inaugurated by [116]. Reproduced with permission from [111].

DIW is the liquid system with the greatest number of dedicated studies available in the literature due to having a simple composition that guarantees reproducibility, limits the number of chemical reactions taking place and facilitates its characterization. Computationally, on the other hand, water composition can be more easily set to represent pure water or to account for compositions that are more complex as in DIW, buffered solution or even for the presence of amino acids.

Chemical reactions induced by plasma treatment of water can be classified into four categories: (i) acid–base reactions; (ii) oxidation reactions; (iii) reduction reactions; (iv) photochemical reactions [71]. Acid–base reactions are associated with the release of hydrogen ions, H^+, in water and are responsible for the change of the pH of the solution, which is a parameter with a direct influence on the liquid conductivity and on several chemical reactions happening in plasma treated

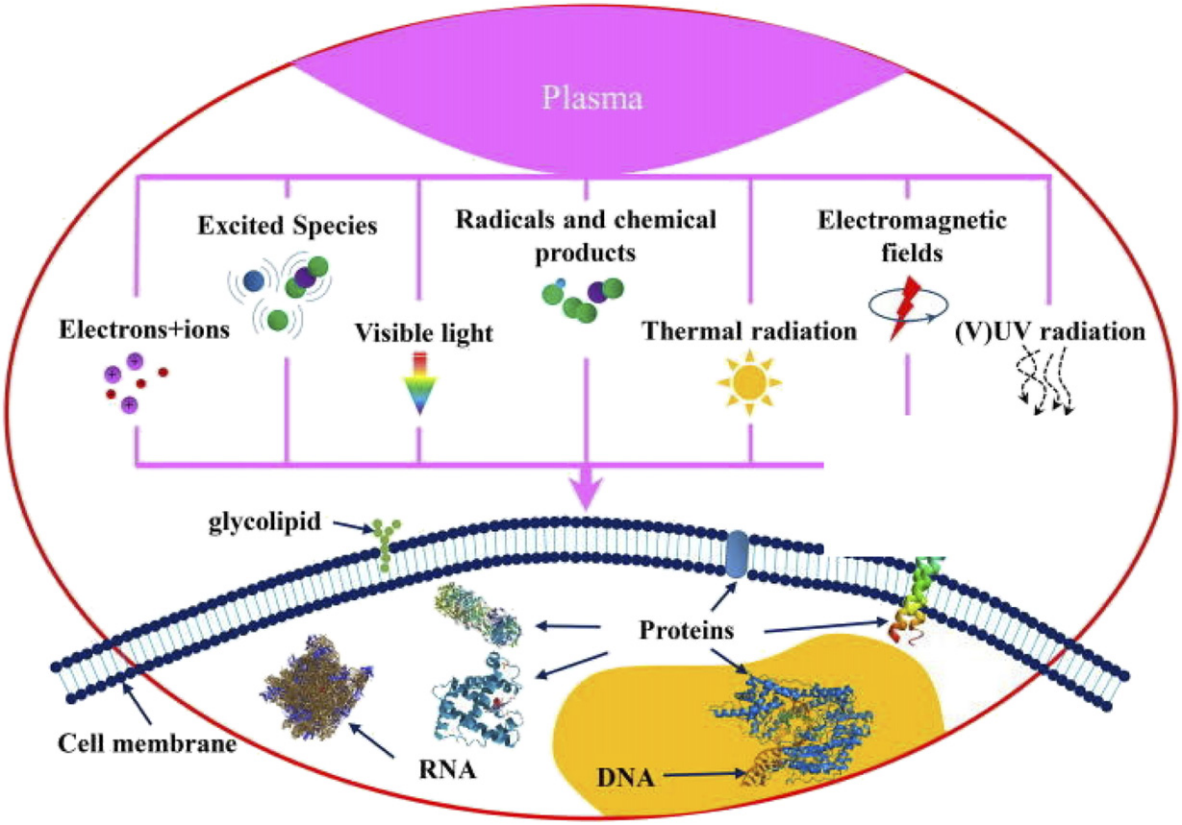

Figure 22. Major targets of CAP compounds on cellular structures with ROS and RNS (radicals and chemical products) as dominant active compounds. Reprinted from [133], with the permission of AIP Publishing.

liquids [71, 75, 76]. Generally, most of the ROS and RNS produced in the plasma discharge display acid–base properties. Which plasma species influence the liquid acidity the most depends on the treatment setup and conditions. In the configuration typically encountered in plasma medicine applications, which involves a plasma discharge in air or surrounded by air, the treated DIW display an increase of acidity mainly due to the formation of nitrous acid, HNO_2, and nitric acid, HNO_3, in the liquid [77]. The presence of these acids is directly related to their diffusion from the gas phase into the liquid phase, favored by their high solubility in water [78]. Their formation in the gas phase is attributed to the following reactions [79], where nitrogen oxide, NO, hydroxyl radicals, OH, and ozone, O_3, are chemical species characteristic of plasma discharges in presence of air and humidity (M is an additional partner, e.g. N_2):

$$NO_g + OH_g + M \rightarrow HNO_{2g} + M \quad (1)$$

$$NO_g + O_{3g} \rightarrow NO_{2g} + O_{2g} \quad (2)$$

$$NO_{2g} + OH_g + M \rightarrow HNO_{3g} + M. \quad (3)$$

Once in the liquid phase, nitric and nitrous acid are hydrolyzed as per the following reactions, leading to a decrease of pH of DIW [80, 81]:

$$HNO_{2aq} \rightarrow H_3O^+{}_{aq} + NO^-_{2aq} \quad (4)$$

$$HNO_{3aq} \rightarrow H_3O^+{}_{aq} + NO^-_{3aq}. \quad (5)$$

Strong acids, such as nitric acid, are completely dissociated in the solution. On the other hand, the dissociation of weak acids, such as nitrous acid, depends on their acid dissociation constant (K_a) and on the pH of the solution. More specifically, the pK_a (defined as $-\log(K_a)$) of the acid represents the approximate value of pH of the solution above which the acid is mostly dissociated.

Oxidation reactions are associated with the presence of oxidizing species in the liquid, either diffused from the gas phase or directly formed in the liquid phase. In the context of plasma-medical applications, oxidizing species serve a dual role of (i) participating in the chemical reactions that define the chemical composition of the liquid, and (ii) directly interact with the biological cells and tissues to be treated. For the case of a plasma in air, the key oxidizing species are OH radicals, H_2O_2, O_3, and peroxynitrous acid, ONOOH, with its conjugate base peroxynitrite, $ONOO^-$. Of these species, O_3 is formed in gas phase and diffuse in the liquid, while ONOOH is formed directly in liquid phase; OH radicals and H_2O_2 presence in the liquid is due to both diffusion after formation in gas phase and direct formation in liquid phase [82, 83].

Similar to oxidation reactions, reduction reactions are linked with the presence of reducing species in the liquid. The superoxide radical, O_2^-, formed either by the hydrolysis of perhydroxyl radicals, HO_2, or by solvated electrons [83], is particularly relevant in plasma medicine applications due to its participation in reactions resulting in the production of peroxynitric acid, O_2NOOH and peroxynitrous acid, ONOOH,

which are considered among the responsible for the biological effects observed for plasma treated DIW [84, 85].

The last class of reactions are driven by plasma-produced UV radiation that penetrates the liquid and induces photochemical reactions. These reactions often result in the production of OH radicals, which have been previously mentioned as strong oxidizers. H_2O_2 photolysis can be taken as an example [86]:

$$H_2O_{2aq} + h\nu \rightarrow 2OH_{aq}. \quad (6)$$

It should also be noticed that, in the case of direct treatments, the part of UV radiation not absorbed by water can directly react with the biological tissue.

Moving beyond the general classification of the reactions that can take place in the plasma-treated liquid, many experimental and computational studies have shed light on the most relevant reactions and on the spatial-temporal distribution of the chemical species in the liquid phase [70, 82, 83]. Liquid phase chemistry is initiated by plasma produced species that diffuse from the gas phase. This process is dominated by the concentration of said species and by their Henry's constant (see table 1). As a further specification, the concentration of these gas phase species depends on many factors, among which type of plasma source is used, the type of electrical excitation, the composition of the gas phase and the duration of the plasma treatment, since the concentration of long living species such as O_3 and HNO_3 increases over time. The behavior of each aqueous species is then determined by diffusion and competitive production–destruction mechanisms, whose relative importance depends on the dissolution depth, meaning that the reactions at the gas–liquid interface differ from those taking place in the bulk liquid [82].

Of the species diffusing into the liquid, positive ions only influence the pH of the solution by charge exchange with water molecules; charged water molecules, H_2O^+, then form hydronium ions, H_3O^+, upon reacting with other water molecules, decreasing DIW pH [83]. On the other hand, electrons diffusing into the liquid rapidly solvate and lead to the formation of OH and O_2^- according to the following reactions:

$$e + H_2O_{2aq} \rightarrow OH_{aq} + {OH^-}_{aq} \quad (7)$$

$$e + H_2O_{aq} \rightarrow {H_2O^-}_{aq} \quad (8)$$

$${H_2O^-}_{aq} + O_{2aq} \rightarrow H_2O_{aq} + O^-_{2aq}. \quad (9)$$

Among the neutrals diffusing into the liquid phase, HNO_3 shows a peculiar behavior since it only affects the solution pH by releasing hydrogen ions (reaction 5). Since neither HNO_3 nor its conjugate base take part in other chemical reactions, nitric acid is not an initiator of the chemistry of plasma treated liquids. It is nonetheless an extremely important species since the solution pH determines acid–base reactions for weak acids such as HNO_2, ONOOH and O_2NOOH. As a further implication of its chemical stability, HNO_3 concentration at different depths depends mostly on mass transport.

More often, neutrals diffusing from the gas phase behave as initiators of liquid chemistry. As a first example, upon diffusing into the liquid H_2O_2, typically the ROS with the highest concentration in liquid phase is dissociated in OH radicals by photons (reaction 6) or solvated electrons (reaction 7) and can take part in the following reaction that results in the production of HO_2:

$$H_2O_{2aq} + OH_{aq} \rightarrow HO_{2aq} + H_2O_{aq}. \quad (10)$$

While reactions 6 and 7 can take place only at the interface, reaction 10 is relevant also in the bulk of the liquid, where the presence of H_2O_2 is guaranteed by its lifetime and enabled by mass transport. As a final note, it was experimentally demonstrated that the H_2O_2 found in liquid phase after plasma treatment is mostly produced in gas phase [73, 87]. Besides its high solubility (indicated by a high Henry's law coefficient, see table 1) parameters like the gas–liquid interface surface area, the gas flow rate as well as the treatment time are relevant for the H_2O_2 transport into the liquid phase so that its concentration is mainly determined by its depletion from the gas phase [88].

Another gas phase species acting as chemistry initiator once diffusing into the liquid is HNO_2, which partially hydrolyzes with the formation of its conjugate base ${NO_2}^-$ and triggers the following chemical reactions resulting in the production of ONOOH and O_2NOOH:

$$HNO_{2aq} + OH_{aq} \rightarrow H_2O_{aq} + NO_{2aq} \quad (11)$$

$$NO^-_{2aq} + OH_{aq} \rightarrow {OH^-}_{aq} + NO_{2aq} \quad (12)$$

$$NO_{2aq} + OH_{aq} \leftrightarrow ONOOH_{aq} \quad (13)$$

$$NO_{2aq} + HO_{2aq} \leftrightarrow O_2NOOH_{aq}. \quad (14)$$

Reactions 11, 12 and 13 require the presence of OH radicals, whose concentration at the interface depends on OH diffusing from the gas phase (75%) and H_2O_2 dissociation (25%), as per reactions 6 and 7 [83]. Due to their chemical reactivity, OH radicals are rapidly depleted and concentrations smaller than 1 nM are found below 1 μm from the interface [82]. Nonetheless, OH radicals participate in chemical reactions also in the bulk of the liquid, where they are released by the dissociation of ONOOH (the reverse of reaction 13, favored under acidic conditions), acting as donor for OH. Since the liquid bulk is lacking the main source of OH radicals present at the interface (diffusion from the gas phase), OH concentration in the bulk becomes negligible because the locally formed OH react and are depleted over timescales as short as microseconds [89]. Peroxynitric and peroxynitrous acids diffusing from the interface are also the source of NO_2 radicals in the bulk of the liquid.

To conclude the overview of the most important ROS and RNS found in plasma-treated DIW, ozone is a species with a very high oxidation potential that can diffuse from the gas phase into the liquid phase. Its concentration in the liquid phase depends mainly on its gas phase concentrations, thus on the operating and environmental conditions of the plasma treatment. Depending on its concentration, O_3 can diffuse outside the interface, reaching the bulk of the liquid. Even if not directly related to the case of plasma-treated DIW, it must be

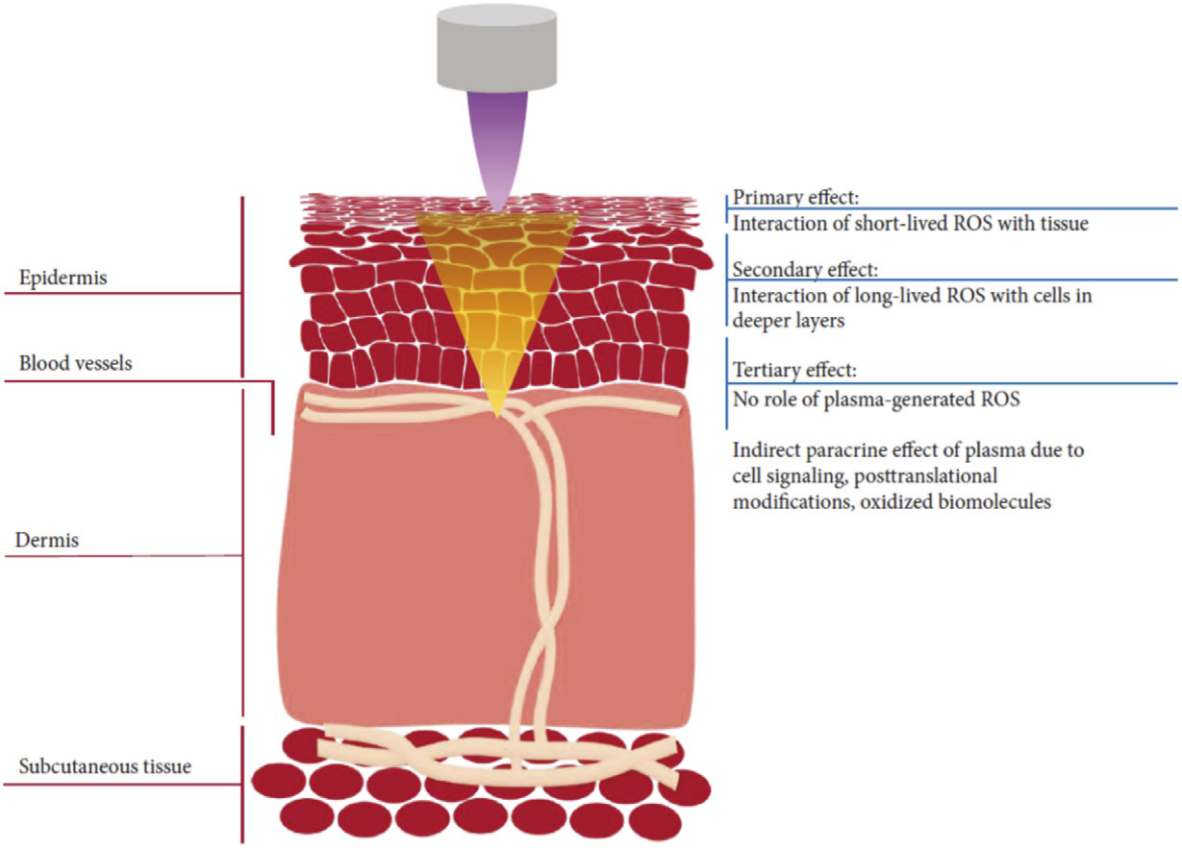

Figure 23. Discussed mechanisms of plasma-caused tissue effects; Reproduced from [134]. CC BY 4.0.

mentioned that in liquids with neutral or basic pH, O_3 in liquid can initiate a set of reactions resulting in the formation of OH radicals [90].

Most of the processes here described are graphically represented in figure 18; continuous and dotted lines represent relevant and negligible reaction pathways, respectively; hollow arrows indicate which species can diffuse from the interface to the bulk of the liquid.

As previously mentioned, chemical reactions continue to take place in the liquid even after the plasma treatment and depend on the pH of the solution. While species such as H_2O_2, NO_2^-, NO_3^- and O_3 can be measured in the DIW hours after the plasma treatment, their concentrations evolve over time. This is shown in figure 19 where data refer to a solution with pH 3.3 [84]; peroxynitric and peroxynitrous acids also can be found in the treated water for a substantial amount of time after the plasma treatment [84, 85, 91, 92].

These long living species are responsible for the release of highly reactive short living species in the liquid, such as the radicals OH, O_2^- and NO_2, ensuring their presence in the liquid even once the plasma treatment is over. In principle, these processes are similar to those happening in the bulk of the liquid and described by the reverse of reactions 13 and 14; HO_2 radicals produced by O_2NOOH decomposition can hydrolyze, with the production of O_2^- radicals:

$$HO_{2aq} \rightarrow H_3O^+_{aq} + O^-_{2aq}. \quad (15)$$

The temperature of the liquid is another parameter influencing post discharge reactions, as shown for the case of peroxynitric acid chemistry by Ikawa *et al* [85], who indirectly demonstrated that the lower is the conservation temperature of the plasma treated liquid, the longer is the time extent over which O_2^- radicals are released. This aspect has obvious practical repercussions, potentially enabling to extend the functional lifetime of a plasma treated solution through freezing and proper conservation.

For interested readers, a more detailed discussion of the post-plasma treatment chemical reactions can be found, among others, in [83–85].

3.2. Plasma-induced chemistry in complex liquids

The liquids encountered within the context of medical applications of plasmas are rarely, if ever, as simple as DIW. In the case of indirect treatments, the choice of the liquid to be treated is guided by reasons such as its biocompatibility and compliancy with regulations. Examples of such reasoning can be found both in the treatment of cell culture media in the context of fundamentals studies on the effect of plasma treated liquids on cells and in the treatment of Ringer's lactate solution with the intent of a clinical application as anti-tumor strategy [93–96]. In the case of direct treatments, biological liquids such as blood and interstitial fluids have complex compositions, with the presence of biomolecules, particles and

buffering species mixed in water. All these components participate in the ongoing chemistry of the plasma-treated liquid, leading it astray from the simple DIW case, and pose significant challenges in the measurement of the reactive species and in the identification of the leading reaction mechanisms. Many groups have started venturing into this highly interdisciplinary field, defining important milestones while struggling with experimental and modelling constraints.

Arguably, the largest number of studies dedicated to complex liquids focuses on the implications of the presence of a buffer compound, a weak acid and its conjugate base, in the treated solution. Once a strong acid or base is added, buffer solutions resist the variation of pH since the equilibrium between the buffer acid and its base is shifted, limiting the increase (in case of adding a strong acid) or decrease (in case of adding a strong base) of hydrogen ions, H^+, in the mixture. Referring to biological liquids, the pH of blood and interstitial fluid is regulated by the bicarbonate buffer system, whose components are carbonic acid, H_2CO_3, and bicarbonate ions, HCO_3^-, and by the phosphoric acid, H_3PO_4, buffer system; proteins (e.g. hemoglobin and albumin) also contribute to maintain the blood pH in the range 7.35–7.45 [97, 98]. Experimental studies on this subject often involve the use of phosphate buffered solutions, where the pH buffering derives from sodium and potassium salts that, mixed in the liquid, originate phosphoric acid and sodium hydroxide (a base). In one of the most thorough articles on the subject [84], Lukes *et al* clearly evidenced that the pH of the solution has a strong impact on the concentration of species formed in the liquid during the plasma treatment and on their evolution in the post-discharge period. With respect to an acidic solution, as could be non-buffered DIW when plasma treated in presence of air, solutions with a neutral pH, such as buffered DIW, result in higher concentrations of H_2O_2 and NO_2^-. In addition, the concentration of these species was found to remain constant for an extended amount of time in the case of a neutral solution, while in acidic conditions they tend to decrease, as previously shown in figure 19. Beside the concentration of long living species, the pH also affects the chemical pathways taking place in the solution. As an emblematic example, ONOOH exists in its acid form (peroxynitrous acid) when the pH of the solution is below 6.8, while above that threshold it will be mostly present in its dissociated form of peroxynitrite ion, $ONOO^-$. Both these species can participate in oxidative reactions, but the character of ONOOH to donate OH radicals and NO_2, represented by the reverse of reaction 13, can only be observed under acidic conditions.

The desire to investigate the treatment of liquids already in use clinically have spurred the interest for Ringer's lactate, a mixture of L-sodium lactate, potassium chloride, KCl, sodium chloride, NaCl, and calcium chloride, $CaCl_2$, in water. Compared to the treatment of DIW, the presence of L-sodium lactate in the solution was found to increase the concentration of H_2O_2 and to induce the formation of acetyl, CH_3CO, and pyruvic acid-like groups, $CH_3COCOOH$ [96]. While this study enabled the identification of which of the components of the Ringer's lactate is responsible for the anticancer properties of the treated solution, detailed descriptions of the chemical composition of the liquid and of the chemical reactions triggered by the plasma treatment are still unavailable.

As a final example, the effect of the presence of biomolecules in the treated liquid was shown by Lietz and Kushner [83] comparing the concentration of reactive species in a treated liquid with or without the presence of peptydoglican. The highlight of their modelling study, reported in figure 20, is that biomolecules can significantly deplete ROS such as O_3 and OH, with opposite impacts on the concentration of molecules that would be consumed by reactions with those species (e.g. HO_2) and others that would need those species to be formed (e.g. ONOOH). The counterpart of this loss of reactivity is the formation of biomolecules with broken bonds and radical sites that can trigger specific chemical pathways that were mentioned, but not covered, in the paper.

3.3. Diagnostic methods and modelling tools for the investigation of plasma-treated liquids

The previous paragraphs should have clarified that much is known of the chemistry initiated in liquids after plasma treatment, and that even more is still unknown. There are many tools available to further explore this scientific field, among which are colorimetric assays, chromatography and EPR spectroscopy; these techniques have been explained in detail elsewhere [70, 99, 100] and it is our intent to provide here only a brief introduction to a few of the available instruments.

Probably the simplest method to assess the concentration of long-living reactive species, such as H_2O_2, NO_2^- and NO_3^-, in the liquid is the use of test strips [74]. When immersed in the liquid for a specified amount of time, test strips change color depending on the concentration of the reactive species they are calibrated for; the operator can thus perform a semi-quantitative measurement comparing the color of the strip against a calibrated chromatic scale provided by the strip manufacturer. However, it has to be kept in mind that test strips might not have a sufficient selectivity as well as accuracy because of cross-sensitivities to other species. Consequently, in plasma-treated liquids that are complex cocktails of species, a distinct identification or quantification of reactive species could be problematic. Therefore, test strips should rather be used for orienting investigations.

On the other hand, quantitative measurement can be obtained using colorimetric assays, which rely on the change of color induced in the solution by a known reaction. Practically, two main approaches are available: (i) dyeing the solution with a compound known to selectively react with a certain species and measuring the discoloring of the solution, as in the case of O_3 bleaching of blue indigo dye [101]; (ii) adding a compound known to selectively react with a certain species with the production of a colored chemical species, e.g. the titanyl sulfate complex (yellow) formed by the reaction of H_2O_2 with titanium sulfate [102]. Depending on the specificities of the assay, these measurements are performed either with a spectrophotometer or a spectrofluorometer.

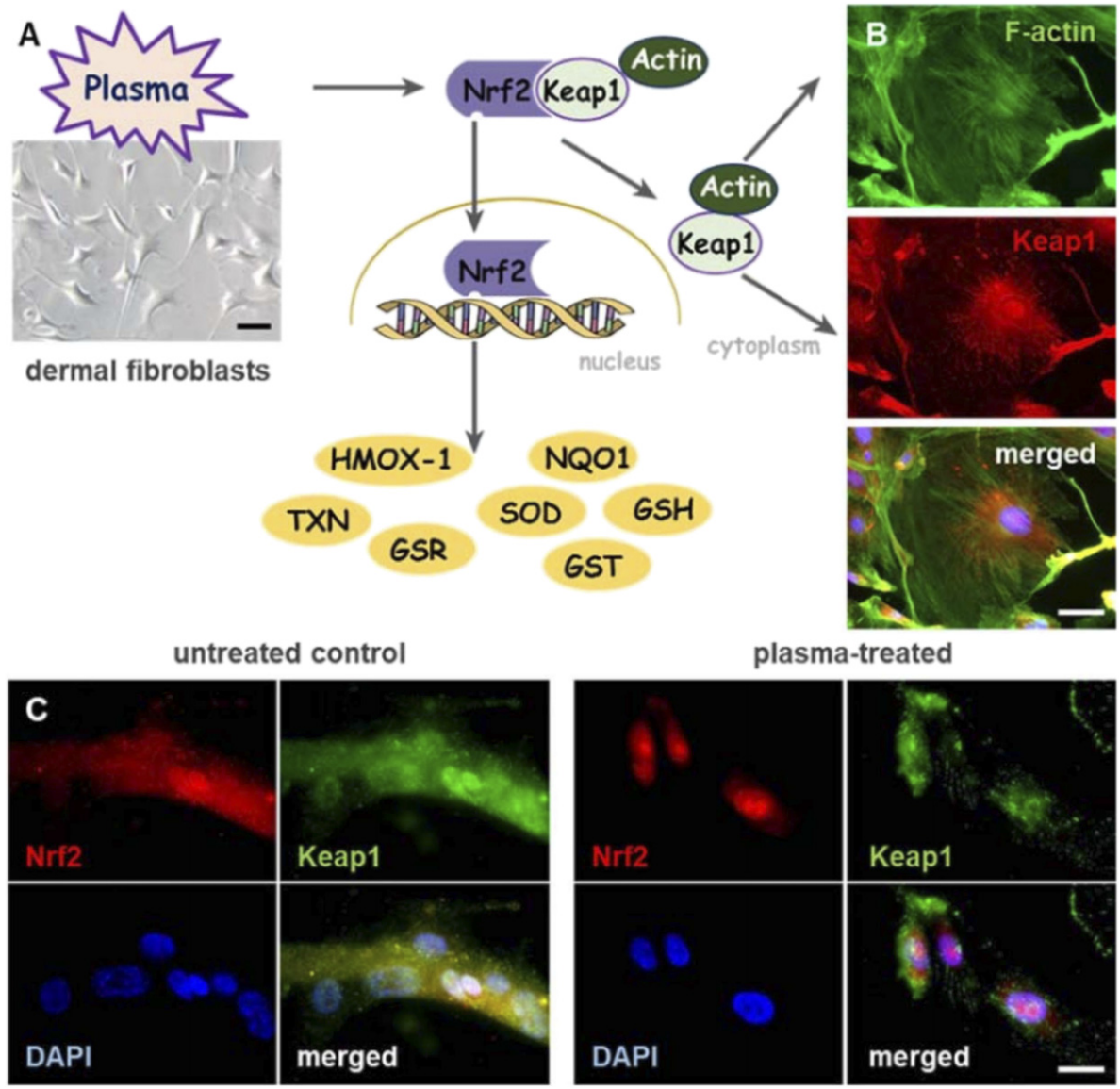

Figure 24. Cold physical plasma triggers nuclear translocation of Nrf2, and induces colocalization of Keap1 with actin filaments in the cytoplasm. (A) Dermal fibroblasts (bright field image, left) were isolated from SKH1 mouse skin and exposed to cold physical plasma-derived ROS/RNS. Upon nuclear translocation of the nuclear factor erythroid 2-related factor 2 (Nrf2), plasma significantly altered antioxidant and phase II detoxification enzymes and proteins (e.g., heme oxygenase 1 (HMOX-1), NADPH quinone oxidoreductase 1 (NQO1), thioredoxin (TXN), glutathione reductase (GSR), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione (GSH) etc). (B) Cytoplasmic localization of Kelch-like ECH-associated protein 1 (Keap1) was detected immunohistochemically by anti-Keap1 antibody (red). Colocalization of Keap1 with actin filaments was visualized by staining with fluorescein isothiocyanate (FITC)-phalloidin (green). (C) Subcellular localization of Keap1 (green) and trans-localization of Nrf2 (red) from the cytoplasm to the nucleus were detected immunohistochemically by anti-Keap1 and anti-Nrf2 antibodies in plasma-treated (right panel), but not control fibroblasts (left panel). Scale bars 100 μm (A), 50 μm (B) and (C). Reproduced from [156]. CC BY 4.0.

Chromatographic techniques rely on a controlled separation of the components of the sample to be analyzed, which flows through a stationary element; the components of the sample that display different affinities for the material of the stationary element flow at different velocities, effectively becoming separated before reaching a detector. In the context of characterizing plasma-treated liquids, ion chromatography has been used to measure NO_2^- and NO_3^- [84] and liquid chromatography has enabled the measurement of short-living species, such as OH and HO_2 radicals, stabilized by using chemical probes [103]. Similarly, short living radicals, e.g. OH and O_2^-, can be measured quantitatively by EPR with the use of spin trapping probes [104–107].

As a final note on the experimental methods, well established techniques relying on the use of standard electrode systems are available for those situations where it is sufficient to measure the redox potential of the plasma treated liquid [108].

Modelling tools offer a complementary and interdependent approach to diagnostic techniques. Indeed, on the one hand, modelling enables the investigation of aspects otherwise impossible to study, such as the identification of the chemical pathways taking place in a plasma-treated liquids; on the other

hand, modelling requires input data, often provided experimentally, and experimental validation. Modelling the plasma treatment of a liquid requires the coupling of three main components: (i) the gas phase model, accounting for plasma formation, plasma kinetics and fluid-dynamic aspects; (ii) the liquid phase model, where transport processes and chemical reactions are simulated; and (iii) the interface model, which takes into account the transport of species from the gas phase into the liquid phase and vice versa. A thorough introduction to the modelling of the plasma treatment of liquids, covering the available approaches and the limitations to consider, is provided in the 2016 plasma–liquid interactions roadmap [70]. Other valuable insights to further approach and better understand the potential of this instrument can be found in [61, 72, 82, 83].

4. Consequences of plasma treatment in cells and tissues

4.1. Redox biology as scientific basis of plasma medicine

As was pointed out already in the previous chapters, ROS and RNS are considered the most important components of CAP, responsible for several biological effects. This fundamental insight was summarized and continuously updated especially by Graves [2, 109, 110]. It has opened up the door to the well-established field of redox biology to explain and interpret several biological effects caused by CAP, leading to the classification of plasma medicine as a field of applied redox biology [111]. The role of redox-based effects in plasma medicine was initially demonstrated indirectly when the presence of antioxidants or scavenger substances like N-acetylcysteine (NAC), glutathione, or ascorbic acid (vitamin C) were able to reduce or completely extinguish CAP-based biological effects in a number of *in vitro* experiments using cultivated cells.

For ROS and RNS, the collective abbreviation RONS is used, too. Moreover, because RNS usually contain oxygen, it is also useful to take the abbreviation ROS for both ROS and RNS. Finally, there are other reactive species in the physiological context like reactive chlorine species (e.g. hypochlorite, ClO^-), or reactive sulfur species (RSS) that can also ROS and RNS include free radicals, i.e. molecules with a single unpaired electron, and nonradical molecules. Among the most important ROS, hydroxyl radical, OH, or superoxide, O_2^-, are radicals, whereas hydrogen peroxide, H_2O_2, or singlet delta oxygen, $O_2(^1\Delta)$, are nonradicals. The most important RNS, nitric oxide, NO, is a radical. Chemically seen, reactions of substances like ROS and RNS involve the transfer of an electron. In these so-called redox reactions, the loss of an electron means that the substance is oxidized whereas the gain of an electron means that the substance is reduced. The tendency of a substance to be oxidized or reduced, respectively, is quantified by its redox potential. According to this concept, it depends on the respective reaction partners and their relative redox potentials whether a single substance will be oxidized or reduced. Redox potentials of ROS and RNS important in redox biology and plasma medicine are tabulated in [112].

The central role of redox processes in cell biology is expressed by statements like 'redox biology is at the heart of life sciences' [113], or 'redox chemistry constitutes the universal language that enables communication between different entities and layers of regulation' [114].

For a long time, ROS and RNS were seen solely as damaging agents and drivers of diseases and aging. Nowadays it is well known that they also play an important role as regulatory mediators in signaling processes and as signaling molecules for other physiological functions. It was realized that redox regulation of cellular processes seems to be equally important as the well-known cellular mechanism of phosphorylation and dephosphorylation of proteins, whereby both processes are interconnected [115]. Generation of ROS and RNS in cells is counterbalanced physiologically by antioxidative mechanisms resulting in a cellular 'redox homeostasis'. Simply spoken, at 'physiological' levels ROS and RNS activate signal pathways and initiate physiological processes. At higher, 'supraphysiological' levels damaging processes are caused. The latter situation was initially named 'oxidative stress', which is 'an imbalance between oxidants and antioxidants in favor of oxidants, leading to a disruption of redox signaling and control and/or molecular damage' [116]. It is one hypothesis that the induction of oxidative stress resulting in a controlled generation of ROS and RNS in the extracellular space was evolutionary and developed as part of the innate immune system as a defense mechanism against bacteria [117, 118]. Generally, oxidative stress can be generated endogenously by cell metabolism but can also result from exogenous influences. However, the stimulating or damaging activity of ROS and RNS has no primarily quantitative aspects but significantly depends on parameters like the specificity and selectivity of ROS and RNS and the compartmentalization of its production or occurrence inside the cell [119].

One of the central questions of redox biology—and so of plasma medicine—is the question of the specific chemistry of redox-based biological processes [113]. The specific analytics of ROS and RNS and its effects on biological molecules and structures is an ongoing challenge both in redox biology and plasma medicine. According to the actual state of knowledge, different ROS and RNS have different selectivity in their reactivity with biological structures.

Among the ROS, the hydroxyl radical, OH, is the most reactive which reacts with most compounds. In living tissue, it is typically generated from H_2O_2 catalyzed by ferrous ions (Fenton reaction). Because of the high reactivity, its lifetime in a biological environment is short ($\sim 10^{-9}$ s). Superoxide, O_2^-, is produced from molecular oxygen, O_2, as a byproduct of mitochondrial respiration (respiratory chain reactions). It is much less reactive, more selective, and has, consequently, a lifetime of few seconds. It reacts in a radical–radical reaction with NO, forming $ONOO^-$, which is a highly potent oxidant and RNS with a half-life of several seconds. Superoxide is rapidly transferred into H_2O_2 by superoxide dismutases (SODs) or in a self-dismutation reaction with another superoxide molecule. Hydrogen peroxide is relatively stable with a half-life of months if it is stored in the absence of light and trace metal contamination. It has a relatively high selectivity

towards proteins containing thiol (-SH) groups. The enzyme catalase transfers H_2O_2 into oxygen and water. Singlet delta oxygen, $O_2(^1\Delta)$, is a photo-excited ROS having a half-life of around 10 μs. It reacts rapidly but selectively with histidine and cystein groups in proteins, but also with unsaturated lipids and partially with nucleic acids. The most important RNS, nitric oxide, NO, is synthesized by nitric oxide synthases utilizing L-arginin as the substrate. It regulates several biological processes including both intracellular and intercellular signaling and is part of defense reactions of macrophages.

One way to classify the activity of ROS and RNS is its differentiation into primary and secondary species. Primary species are the superoxide radical, hydrogen peroxide, and nitric oxide, whereas the hydroxyl radical and peroxynitrite can be seen as secondary species resulting from reactions with primary species or a transition metal. There are some hints that primary species in most cases reversibly react with the target molecules and are, therefore, more associated with signaling processes. Primary species are well controlled, e.g. by enzymes like SOD, or catalase and have only a weak damaging potential. Secondary species are worse controlled and mainly cause irreversible reactions and are therefore, more associated with damaging processes. It is hypothesized that—against the background of evolution—primary species are mostly responsible for physiological signaling whereas secondary species serve for extracellular actions, e.g. killing of bacteria, and are, at the same time, also able to cause damage to the cell [118]. Generally, the primary species superoxide and hydrogen peroxide are seen to be the major signaling molecules in cell physiology. However, this seems to be a very superficial and only orientating categorization.

A crucial mechanism of redox signaling is based on the oxidation of protein sites containing cysteine which has a thiol (-SH) group, mainly by hydrogen peroxide or superoxide. Oxidation of the thiol group to the sulfenic form (-SOH) results in allosteric changes of the respective protein and subsequently in altering is function (e.g. activation vs inactivation). Enzymatic reduction of sulfenic groups back to the thiol group by thioredoxins or glutaredoxins can make this protein modification reversible. Higher levels of ROS may further oxidize thiol groups to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) residues. These reactions are irreversible and lead to permanent protein modification or damage. This is a simple example to demonstrate the balance between redox signaling on the one hand and damaging oxidative stress on the other, in living systems, by regulation of both the intracellular ROS level and the locally available enzyme activity. For more insight into the cysteine-based redox signaling, see [120, 121].

ROS and RNS can be produced by various endogeneous and exogeneous sources. The intracellular steady state is controlled by enzymatic (e.g. SODs, catalases, peroxidases) and nonenzymatic (e.g. small-molecular weight substances like vitamin E, vitamin C, glutathione, ubiquinone, beta-carotene) antioxidant mechanisms and adaptive responses leading to antioxidant gene expression. Following the rapid increase in knowledge of redox biology in recent years leading to much more insight into the well-balanced cellular redox system, the concept of 'oxidative stress' was reassessed to meet the fact that oxidative impact ('oxidative stress') includes both the redox-caused damage and redox signaling, as well as its sophisticated regulation in living systems. Low, 'physiological' exposure of ROS and RNS facilitates addressing specific targets to induce redox signaling. This state is named 'redox eustress'. High, 'supraphysiological' exposure results in disrupted redox signaling and/or damage of biological structures. This situation is named 'oxidative distress' [116]. It will become apparent that this concept can be well adapted to the different activity of CAP in biological processes (see section 4.2).

In general, redox biology, as well as the concept of oxidative stress and redox homeostasis, is much more complex than can be explained in this paper. For more detailed insight, several review papers and books are available, e.g. [113–115, 117, 119, 122–128]. However, this short introduction to the basic concepts of redox biology should be helpful in understanding the basic principles and mechanisms of biological CAP effects and the consequences for medical applications.

4.2. Fate of plasma-generated ROS and RNS in biological systems

A central question of basic research in plasma medicine was how the biological CAP effects are transmitted into living systems, i.e. how plasma 'components' reach cellular targets and induce biological effects.

Plasma contains highly motile electrons and is conductive. Therefore, it can transfer electrical current to cells and tissue with possible biological consequences [129]. Likewise, emitted electromagnetic radiation, above all, UV light, generally may elicit biological effects [130, 131]. However, with the intensity as these physical plasma components arise in CAP application, their cellular impact should be more or less negligible. Nevertheless, their combined effect during direct plasma treatment, as well as their supporting impact generating reactive species in plasma–liquid interaction, should be kept in mind and needs more research.

An important step to gain deeper insights into biological plasma effects was the discovery of ROS and RNS as the most dominant plasma effectors and that these plasma-generated ROS and RNS are more or less the same as that are acting in regular cell physiology. Consequently, plasma treatment may be understood as a kind of 'local oxidative stress' terming plasma medicine as 'applied redox biology' following the concept of Sies (figure 21) [111, 116].

Another important step was the generalizing statement that all living systems are surrounded by liquid phases, i.e. the primary contact of plasma with living systems might be in most cases a plasma–liquid interaction. This led to intensive investigations into the mechanisms of plasma–liquid interactions (see section 3).

Even if this research led to an important increase in knowledge about the fate of plasma components in biological applications, it has to be taken into account that this research was primarily based on *in vitro* experiments with bulk liquids or cell cultures in which the covering liquid phase is comparatively bulky. In real setups, this situation may be distinctly

different, e.g. in wounds that may be covered by a very thin liquid film, or in the case of dry skin where it is not a liquid film that is the primary target of plasma impact but the *Stratum corneum*, a μm thick protective layer. Consequently, the situation is much more complex and needs more differentiated consideration.

Generally, reactive species generated in the plasma/gas phase (referred to as 'source' in figure 21) have to be transported to the biological target and, subsequently, have to be exposed to biological structures to react with. Initially, the transport and effective range of reactive species from the plasma/gas phase in the biological environment and their subsequent biological impact strongly depends on the extracellular matrix composition (referred to as 'sink' in figure 21). It is necessary to take into account that the extracellular space in living tissue contains proteins, carbohydrates and other components that are potential reaction partners for plasma-generated reactive species. The dimension of the liquid layer around cells—if present—as well as its composition strongly influences the free range of reactive species, which is otherwise determined by their reactivity (see section 4.1 or the review paper [132]).

Provided that there is an albeit thin liquid phase between the plasma/gas phase and the biological target (cell), different processes have to be taken into account [112]: (i) gas phase transport of reactive species as was mentioned in section 2.2; (ii) processes at the plasma/gas–liquid interphase; (iii) processes inside the liquid. At the interface, both dissolvation of plasma/gas phase species into the liquid (see table 1 in section 1) and desolvation of species (above all water molecules) from the liquid into the vapor phase may occur.

Generally, the liquid phase can serve as a transport medium to bring ROS and RNS from the plasma/gas phase to the biological target, or as a reaction medium where ROS and RNS from the plasma/gas phase may react with other molecules of or within the liquid to generate secondary reactive species. Affected both by the reactivity of different reactive species and the presence of potential reaction partners in the liquid phase (cellular environment), the free range of reactive species may be different. It is a consensus that short-lived ROS and RNS rapidly decay in aqueous liquid resulting in more stable species like nitrite, NO_2^-, nitrate, NO_3^-, or hydrogen peroxide, H_2O_2. Therefore, the plasma/gas–liquid interphase has a special role because there is an accumulation of higher-reactive species possible allowing chemical reactions that are not realizable in the bulk liquid. Additionally, photochemical effects of UV may occur in this interphase dependent on the type of plasma discharge and the contact of the plasma with the liquid, which may support the generation and/or conversion of ROS and RNS [70]. Therefore, it can be stated generally that the dimension and the composition of the liquid phase around a biological target may be decisive for the fate of the reactive species from the plasma/gas phase as well as for their biological impact. Hence, any conclusions by analogy from *in vitro* experiments with classical 2D cell culture models to the real situation e.g. in a wound, have to be considered with caution due to the possibly different liquid phase dimensions as well as the different-by-composition cell culture media involved. These effects also have to be taken into account if the biological activity of plasma-treated liquids is considered in comparison to direct plasma treatment (see section 3).

Looking further at the cellular level, the most important biological structures for an impact of ROS and RNS are (i) the cell membrane comprising a phospholipid bilayer containing cholesterol, proteins, and glycolipids; (ii) intracellular proteins; and (iii) nucleic acids (DNA, RNA) inside the cell (figure 22). Because of their different localization, accessibility for plasma-generated reactive species should be different, too.

The cell membrane can be seen as the key target for the impact of plasma-generated ROS and RNS. Some reactive species such as ozone, nitric oxide or atomic oxygen are able to penetrate biological membranes; others cannot because of their polarity, e.g. singlet delta oxygen, hydroxyl radical, superoxide anion, nitrite, or peroxynitrite. Hydrogen peroxide is known to be able to diffuse into the cell via protein channels called aquaporins. Moreover, cell membrane permeability and fluidity can be further increased or modulated by plasma treatment via a decrease of the mechanical strength and improvement of pore formation based e.g. on lipid peroxidation. This can be supported by plasma-transmitted electrical fields [133].

Plasma-generated ROS and RNS may penetrate cell membranes or interfere with them, inducing the generation of secondary effects inside the cell. Stimulation of the intracellular production of ROS and RNS can amplify reactive plasma species-based biological effects. In all cases, intracellular redox signal cascades and response mechanisms are activated. There are several reaction chains and mechanisms involved that are as yet only partially deciphered. Privat-Maldonado *et al* [134] give more details on plasma effects on cellular membranes and their consequences for cell physiology.

As was pointed out already, all these hypotheses are more or less based on the model representation that there is a simple system containing a plasma/gas phase, a liquid phase (with different roles of the plasma/gas–liquid interphase and the bulk liquid), and the cell, with the cell membrane as the primary target of impact of plasma-generated compounds. Even if this simple model was the basis of important progress in understanding biological plasma effects, it cannot completely explain some plasma effects in deeper layers of tissue where a direct plasma impact must be considered as unlikely. Early reported examples of biological depth effects after plasma treatment of intact skin were an increased oxidation of beta-carotenes (which are part of the antioxidant network of the human skin) or an increased number of proliferating cells in deeper skin layers [135, 136]. Moreover, increased tissue oxygenation and blood circulation were found after both plasma treatment of intact skin [137–140] and skin wounds [141–143]. Even if for the latter effects physical phenomena like plasma-transmitted electrical fields may be at least partially responsible, in general, such effects in deeper skin and tissue layers cannot be explained completely by direct plasma action.

Generally, there are three mechanisms under discussion which may explain plasma effects in deeper skin and tissue layers: (i) diffusion of plasma-originated or secondary generated long-lived ROS and RNS into deeper tissue layers; (ii) action of proteins with plasma-caused posttranslational modifications or other oxidized biomolecules; and (iii) stimulation of cell–cell communication after direct exposure of surface cells to plasma or plasma-generated species, respectively (figure 23).

More so than in plasma–liquid interaction, it is challenging to follow and detect trajectories of ROS and RNS inside tissues. Therefore, several more or less complex *in vitro* models for tissue penetration of plasma-generated species were used and complemented by computational methods. For details on these approaches, see the review papers [134, 144–146]. In these models, penetration depths of plasma-derived ROS and RNS from 5 μm up to several mm were proposed, dependent on the models used, experimental conditions, as well as evaluation methods.

Furthermore, oxidative modifications of organic molecules were identified to play an important role in the transmission of biological plasma effects. Here, cysteine thiol groups are important target structures [147, 148], not only for redox-based signalling as mentioned in section 4.1. It was also found by *in vitro* experiments that plasma-treated cysteine might exert distinct biological effects in cells [149]. Such modifications of cysteine groups as well as other plasma-induced posttranslational peptide or protein modifications [150, 151] may be responsible for prolonged secondary effects of plasma treatment also in deeper tissue layers.

Finally, because a diffusion of plasma-generated ROS and RNS, as well as secondary species generated inside the tissue into deeper skin and tissue layers might also be limited because of their limited range in a biological (reactive) environment, so-called 'bystander' or paracrine effects should give another satisfactory explanation for depth effects [2, 109, 134]. A very early idea of possible cell–cell communication based on molecules secreted by plasma-treated cells was given by Kalghatgi *et al*, who demonstrated *in vitro* that endothelial cells treated with plasma release the fibroblast growth factor-2 (FGF2). The FGF2 stimulates proliferation of other cells that are not plasma treated. These experimental results suggested that plasma treatment might enhance endothelial cell proliferation due to reactive species-mediated FGF2 release [152]. Meanwhile, there is incidence from several *in vitro* and *in vivo* experiments that such cellular secretion of growth factors and cytokines is an obvious cellular response to plasma treatment (see section 4.3).

4.3. CAP-triggered biological signaling in oxidative stress response, wound healing and cancer treatment

As was explained before, the decision regarding whether a plasma treatment induces oxidative eustress or distress depends on (i) the amount of ROS and RNS reaching the biological target (determined by their production by the plasma device—'source', and their modulation by the liquid environment—'sink'); and (ii) on the adaptive response of the biological system itself (see figure 20). As was pointed out already (see section 4.1), mammalian cells have a well-balanced system to control the intracellular redox state based on sophisticated redox-controlled reaction chains. The phenomenon of low concentrations of agents having different effects compared to higher concentrations is known as hormesis. It can also be adapted to the ROS/RNS-based biological plasma activity [109, 134].

One of the key translators of redox signalling in cellular response against imbalances in redox homeostasis is the nuclear factor erythroid 2-related factor (Nrf2) pathway [124, 153–155]. Under physiological conditions, Nrf2 is associated with the action-binding Kelch-like ECH-associated protein 1 (Keap1). This binding sequesters Nrf2 in the cytoplasm and leads to its rapid proteasomal degradation to maintain baseline Nrf2 levels with a half-life of 20 min. By redox modification of thiol (-SH) groups of cysteine-residues of Keap1, Nrf2 is released from its complex and translocates from the cytoplasm to the nucleus where it dimerizes with small musculoaponeurotic fibrosarcoma proteins and binds to antioxidant responsive elements (ARE) on DNA. This promotes the upregulation of antioxidant genes, which encode detoxifying enzymes and proteins like glutathione (GSH), glutathione reductase (GSR), glutathione S-transferase (GST), superoxide dismutase (SOD), heme oxygenase 1 (HMOX-1), thioredoxin (TXN), and NADPH quinine oxidoreductase 1 (NQO1) (figure 24).

According to the actual state of knowledge, this pathway is one of the most active regulatory networks also in response to plasma treatment [134]. It was repeatedly demonstrated *in vitro* but also in animal studies *in vivo*, that CAP treatment is able to stimulate this Nrf2 pathway resulting in subsequent activation of Nrf2-ARE targets [157–159]. The fact that the Nrf2 pathway is induced by CAP treatment is a strong support of the concept of CAP-induced oxidative stress response of cells as it is shown in figure 21.

Stimulation of the Nrf2 pathway by CAP treatment can be taken as one of the mechanisms to protect mammalian cells from genotoxic plasma effects. A huge number of *in vitro* studies report on potential genotoxic CAP effects on isolated, naked, or cellular DNA [160]. However, estimation of genotoxic effects of CAP by *in vitro* standard procedures for mutagenicity testing did not result in an extended mutation rate of plasma-treated cells [161–165]. This was confirmed by an animal study using hairless immunocompetent mice [166], and in clinical follow-up investigations of plasma-treated wounds [167, 168], where consistently no precancerous lesions or other signs of skin or tissue alteration where found that could give evidence of cancer formation. For a review on safety aspects of CAP application, see also [169].

Besides its central role in cytoprotection, there is some evidence that activation of the Nrf2 pathway also has a major impact on wound healing. It was reported that Nrf2 activation can stimulate the proliferation and migration of epithelial cells during wound repair. Nrf2-activating compounds are estimated to be a promising therapeutic approach in wound healing [154, 155, 170].

Above all, in an acute wound healing study on mice, an early activation of the Nrf2 pathway was demonstrated *in vivo*

and *ex vivo* in skin tissue, dermal fibroblasts and epidermal keratinocytes [159]. Besides the key role of the Nrf2 system in the upregulation of detoxifying and antioxidant genes, activation of Keap1 stabilizes the architecture of F-actin cytoskeleton and focal adhesion, and increases granulation tissue formation and matrix deposition. As a key regulator in macrophages, Nrf2 mediates the infiltration of macrophages and neutrophils and the upregulation and secretion of pro- and anti-inflammatory ligands, which activate signalling and intracellular generation of ROS and RNS. CAP supports angiogenesis by the recruitment of endothelial cells, growth factor expression like keratinocyte growth factor, epidermal growth factor, or vascular epidermal growth factor, and activates protein kinase B (Akt), which are inducers of Nrf2 expression [111, 143, 156, 159].

Besides the Nrf2 pathway, other proliferative-acting pathways like the MAPK/ERK pathway (mitogen-activated protein kinases/extracellular signal-regulated kinases) or the Hippo signalling pathway are identified in plasma-treated skin cells *in vitro* [134, 171–174].

Because wound healing is a complex and multi-stage process [175, 176], not only proliferative, but also apoptotic effects are necessary to manage the removal of inflammatory cells and to inhibit scar formation of granulation tissue in the later parts of wound healing. Here, as another redox-sensitive transcription factor, the tumour suppressor protein p53 plays a role in the regulation of cell proliferation and apoptosis, but also in angiogenesis, cell cycle regulation and DNA repair [177, 178]. Its activity depends on the state of wound healing and the concentration of reactive species and is linked to the Nrf2 system. At low p53 expression levels, it enhances the Nrf2 protein level, whereas at high p53 expression the Nrf2 mediated cell response is inhibited and cell senescence or apoptosis is supported. It was demonstrated repeatedly that this p53 signalling pathway is also triggered by CAP treatment [111, 134, 179].

Taken together, based on a huge number of *in vitro* results, that are verified *in vivo* in some animal trials, important details of CAP-supported stimulation of tissue regeneration and wound healing were uncovered, mainly leading to [156, 158]:

- Promotion of re-epithelialization and the acceleration of wound closure,
- Reduction of inflammation by activation of cytoprotective mechanisms and by recruiting of immune cells into the wound area,
- Fibroblast activation inducing rearrangement of the actin cytoskeleton and promoting matrix synthesis,
- Activation of wound healing-relevant cytokines and growth factors in fibroblasts and keratinocytes, and
- Induction of neovascularization.

Finally, increased tissue oxygenation and blood circulation caused by plasma treatment, as mentioned above (see section 4.2), are additional factors of wound healing support. Moreover, it was demonstrated repeatedly *in vitro* and *in vivo*, that CAP also promotes blood coagulation; see for example [180–182].

According to the concept of oxidative eustress and distress that was adapted to plasma medicine (see figure 21), support of tissue regeneration by CAP treatment can be assigned to redox-regulated 'oxidative eustress'. Following this concept, a more intensive (longer) plasma treatment resulting in a higher impact of ROS/RNS must lead to an overburdening of adaptive responses of the cell and, consequently, to dysregulation and cell death [183, 184]. Indeed, this phenomenon of the stimulation of cells with shorter plasma treatment time and the inhibition of cellular function up to cell death after longer treatment times was described *in vitro* repeatedly; see for example [136, 185]. That means, on the one hand, that a careful application of plasma is necessary with parameters that are strongly dependent on the specific characteristics of the plasma source used if stimulation effects, e.g. in wound healing, are intended in an optimized manner. On the other hand, CAP can also be used to inactivate cells in a controlled manner.

It is one of the early findings of fundamental research in plasma–cell interactions that CAP is able to inactivate cancer cells. A huge number of *in vitro* experiments, but also first animal experiments, could demonstrate the anticancer effects of CAP; see [12, 186, 187]. This is important as well as relevant for potential medical applications of CAP in the field of cancer therapies because the evasion of apoptosis is one of the hallmarks of cancer [188, 189]. It was proven that different cancer cell lines are very different in their sensitivity to plasma impact [190–192], and that cell type, cancer type and cell culture conditions strongly influence CAP effects, on the one hand [193], but also the tumor microenvironment containing malignant cells, endothelial cells, fibroblasts, tumor vasculature and the extracellular matrix on the other [194]. Nevertheless, it is a current hypothesis that cancer cells are generally more sensitive to CAP treatment compared to nonmalignant counterparts.

ROS/RNS are main contributors for the efficacy of CAP in killing cancer cells [183] because they are able to trigger different specific intracellular mechanisms leading to cell cycle arrest or cell death via necrosis or apoptosis, a form of regulated cell death [195]. Other processes like senescence, an irreversible cell growth arrest in response to stress like oxidative stress and DNA damage, or autophagy, a lysosome dependent cellular mechanism to remove needles or impaired components, were also described.

CAP-induced cell death via ROS/RNS impact is a complex process about which the details are still only partly understood. If increased levels of plasma-generated ROS/RNS occur inside the cell, direct oxidative change of cellular lipids, proteins, or nucleic acids may lead to its general damage or dysfunction. Moreover, a variety of intracellular processes may be influenced, including caspase activation, cell-cycle disruption and disruption of other multiple pathways. For example, apoptosis can be induced by triggering specific pathways like the MAPK/ERK pathway, which is not only a regulator of cell proliferation, but also related to apoptosis. ROS/RNS signalling is also coupled to calcium signalling. Calcium is an important second messenger that is involved in cell life and death decisions. The main intracellular storage of calcium is the endoplasmatic reticulum (ER), where calcium is released

by specific receptors that are sensitive both to ROS and to calcium. Increased ROS/RNS, as well as calcium concentration in cells after CAP treatment, are features of ER stress. A specific connection of ER with mitochondria allows the exchange of calcium, lipids and metabolites. ER stress, e.g. caused by enhanced ROS impact, leads to increased calcium content in mitochondria and subsequent depolarization of mitochondria membrane potential. This in turn causes a release of cytochrome c activating mitochondria-dependent apoptosis. Another pathway resulting in mitochondrial apoptosis is triggered by NO, via blocking cytochrome oxidase, the terminal enzyme of the electron transport chain in mitochondria. These are only some examples of molecular mechanisms of CAP-induced cell death. More details are explained in several review papers, e.g. [134, 186, 191, 194, 196].

As was pointed out already, the balance between oxidative eustress and distress is finally defined by the amount of ROS/RNS inside the cell and its impact on intracellular regulatory mechanisms. Consequently, both stimulation and induction of cell death are possible in principle in any type of mammalian cells. However, the most interesting question is how to explain the predicated higher sensitivity of cancer cells to oxidative distress.

On the one hand, there are generally increased steady-state ROS levels in cancer cells resulting from unique metabolic activities [124, 197]. Therefore, cancer cells might be more vulnerable to further oxidative stress caused by CAP application because the toxic threshold might be reached earlier compared to nonmalignant cells. Additionally, the anti-oxidative defence system based on enzymatic and nonenzymatic mechanisms (see section 4.1) may be different in cancer cells compared to their nonmalignant counterparts.

On the other hand, the enhanced plasma sensitivity of cancer cells is explained by a selective stronger rise of intracellular ROS/RNS caused by specific characteristics of cancer cells. The first barrier that is tackled and has to be overcome by plasma-caused ROS/RNS is the cell membrane. Because of its hydrophilicity, the direct transmembrane diffusion of ROS/RNS is limited. Transmembrane channel proteins called aquaporins serve as water channels facilitating the transport of water between cells but also enabling diffusion of small hydrophilic molecules. An increased expression of aquaporins in cancer cells may be one cause for increased impact of ROS/RNS and resulting in the higher sensitivity of cancer cells to plasma treatment. On the other hand, the diffusion of ROS/RNS through phospholipid membranes depends on the cholesterol content providing membrane stability and fluidity. The amount of cholesterol is often reduced in cancer cells. Lipid peroxidation caused by ROS/RNS may contribute to additional pore generation in such cholesterol-depleted cell membranes. Selected aspects of plasma selectivity to cancer cells in comparison to normal cells are presented schematically in figure 25; see also [2, 190, 198, 199].

A further hypothesis under discussion is based on an enhanced cell-based generation of H_2O_2 via plasma-generated superoxides by extracellular superoxide-dismutase on the cell membrane and a subsequent triggering of immune attack on cancer tissue by H_2O_2-mediated lymphocyte activation [200]. Another redox-regulating enzyme is catalase, which is expressed to a higher extent on cancer cell surfaces to protect them from intercellular ROS/RNS-dependent apoptosis-inducing signaling. Plasma-generated singlet delta oxygen, $O_2{}^1\Delta$, is supposed to inactivate membrane-bound catalase which leads to the generation of tumor cell-derived secondary singlet delta oxygen in a self-perpetuating manner catalyzed by transmembrane NADPH oxidase (NOX) resulting in selective ROS/RNS-dependent apoptosis in tumor cells [201]. All these hypotheses are more or less feasible but currently still under discussion [192].

Finally, it has to be mentioned that substantial *in vitro* and *in vivo* evidence provided that redox-based effects of CAP causing cell death may also have immunomodulation aspects. CAP is able to affect immune cells, which is mainly investigated in conjunction with cancer treatment. There are hints that CAP treatment may lead to systemic tumor-specific immunity [202]. Current results in this field are summarized in [203]. Several studies reported that plasma-treated cancer cells succumb to the so-called immunogenic cell death (ICD) promoting anticancer immune responses, which was observed before e.g. in radiotherapy [204, 205]. ICD is a regulated cell death mechanism aligned with the adaptive immune system. Cells undergoing ICD release so-called danger-associated molecular patterns to communicate with immune cells. This includes active secretion of adenosine triphosphate (ATP) or mobilization of calreticulin (CRT) to the outer cell surface. As a result, so-called antigen-presenting cells (APC), i.e. macrophages and dendritic cells are alerted. Attracted by ATP and finding surface CRT, the cancer cells are phagocytised with the cell fragments serving as antigens. Antigen-loaded APCs stimulate the generation of antigen-specific T-cells and memory cells. As a consequence, these circulating cells can then target other cancer cells of the same origin, e.g. metastatic cells, that are not CAP treated [206–209]. Such ICD processes are one explanation for abscopal plasma effects, i.e. reduction of growth of tumors in animal experiments that were not exposed to plasma treatment but are localized elsewhere in the same animal [210, 211]. This may open a highly promising therapeutic approach for cancer treatment in cases where direct plasma control of a metastatic lesion cannot be realised. Moreover, these immune-modulating effects of plasma may open up novel strategies of vaccination [212], but may also have potential consequences with regard to systemic effects of a local therapy by CAP.

To close this chapter on CAP-triggered biological signalling, it has to be mentioned explicitly that most of the biological effects of CAP are not only caused by direct CAP application but also by application of plasma-treated liquids, sometimes called ‘indirect’ plasma application [213]. This underlines the crucial role of plasma-caused ROS/RNS that generated in or transferred into liquids (see section 3). On the other hand, such plasma-treated liquids, which are often called plasma-‘activated’ liquids (e.g. plasma activated medium, PAM, or plasma activated water, PAW), seem to open up very promising therapeutic approaches above all in cancer therapy [93–95, 214]. However, despite a general equivalence of basic anti-cancer effects, there seem to be some

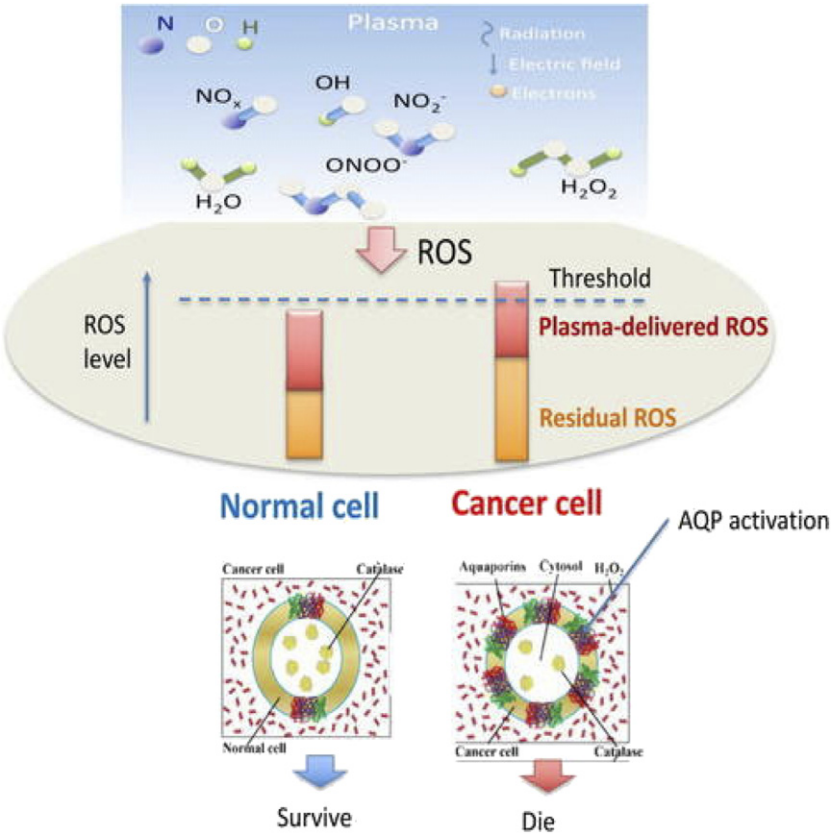

Figure 25. Schematic representation of selected aspect of plasma selectivity to cancer cells. Reprinted from [199],with the permission of AIP Publishing.

differences between direct plasma treatment and the application of plasma-treated liquid. In direct plasma treatment, three plasma related factors may become active: (i) physical plasma components; (ii) short-lived reactive species occurring directly from the plasma impact; and (iii) long-lived reactive species generated in liquid phases. In plasma-activated liquids (PAL), only long-lived reactive species should be active. There are some hints in the literature that both short-lived reactive species and physical factors may be active in some sensitization of cancer cells, finally causing additive or synergistic effects together with any 'basic' activity of long-lived reactive species [215]. Recent reports on anti-cancer effects of CAP only based on physical impact may lead to new insights into the unique characteristics of CAP in biomedical applications [216, 217].

4.4. Cold atmospheric plasma effects on microorganisms

With the improved availability of CAP technology in the 1990s [218], the first ideas to use it for biomedical applications resulted from its strong antimicrobial activity [13, 219]. Meanwhile, there are plenty of reports on CAP inactivation of bacteria including bacterial biofilms, fungi, bacterial spores, and viruses, summarized in review papers such as [220–226].

In contrast to the huge research effort in recent years to elucidate mechanisms of plasma effects on mammalian cells including cancer cells, knowledge of mechanisms of inactivation of microorganisms by CAP is still limited. There are some reports on mechanisms of low-pressure cold plasma interaction with microorganisms which is mainly attributed to UV-based DNA damage in combination with erosion of microorganism structures by UV-based photodesorption and etching processes by reactive plasma species [227–229]. However, these mechanisms are not fully transferable to atmospheric pressure plasmas.

Supposed mechanisms of CAP effects on microorganisms are reported in some review papers, e.g. [14, 219, 221, 223, 230–232]. Here, only a brief summary of the current knowledge in this field will be given.

In general, the antimicrobial effectivity of CAP depends on plasma processing parameters, environmental factors, and properties of the respective microorganisms [219, 223]. Processing parameters include input power, treatment time, working gas type and flow rate, and exposure mode determined by device characteristics. In the end, all these parameters determine the composition and quantity of biologically active plasma components.

Antimicrobial plasma effects are not only promising for therapeutic applications like wound healing or pathogen-associated skin diseases, but also in hygiene and food processing. Therefore, the surface or matrix where the microorganisms are treated may be variable. Above all, humidity is a determining factor, i.e. if the microorganisms are treated in a dry state or in a wet or liquid environment.

Microorganisms' sensitivity to CAP is different and depends not only on microorganism species but also on growth phase and mode of growth. Even if there is no general consensus, gram-negative bacteria with outer membranes of lipopolysaccharides and a thin (∼2 nm) cell wall consisting of peptidoglycan are reported to be more susceptible to CAP than gram-positive bacteria with a thicker peptidoglycan cell wall (∼40 nm) and no outer membrane. Some bacteria like *Bacillus subtilis* are able to pass over from a vegetative to a sporulated state with hard and multi-layered coats; these bacteria spores are hard to inactivate by CAP. Finally, the resistance of fungi to CAP is usually higher, compared to bacteria. Here, the fungal cell wall consisting of chitin, which is more rigid than the peptidoglycan bacterial cell wall, may be critical. Many bacteria and, above all, human pathogens are able to form biofilms, i.e. sessile bacterial communities attached to a substratum or to each other that are embedded in a matrix of extracellular polymeric substances (EPS). These biofilms are highly coordinated communities with a—compared to planktonic bacteria—higher resistance against environmental stress including antimicrobial measures, and are difficult to inactivate or remove using CAP [225].

Similarly to mammalian cells including cancer cells, microbicidal CAP effects are mainly attributed to the activity of ROS and RNS. UV effects as in low-pressure plasma do not seem to be the major inactivating factor because of low UV doses and UV absorption by ambient atmospheric air. Some proposed and mainly ROS/RNS-based inactivation mechanisms of microorganisms are depicted schematically in figure 26.

Antimicrobial CAP effects are mainly attributed to three general actions: (i) permeabilization or damage of cell membrane or wall, (ii) modification or damage of intracellular proteins, and (iii) DNA damage.

There are some hints that there is some balance between biological impact on cellular processes and physical destruction of bacteria dependent on the intensity of plasma application [233, 234].

Damage to the cell membrane or cell wall can be caused by oxidative impact of plasma-generated reactive species on the one hand and, under specific conditions, by electrostatic disruption resulting from accumulation of charged particles generated from plasma on the other. Because of the strong dependence of the effect of the electrostatic forces on the cell radius, microorganism cells may be more susceptible to this physical impact than mammalian cells because the latter are usually one order of magnitude larger. In both cases, a leakage of intracellular components like nucleic acid and protein, but also an increased influx of ROS/RNS into the cell, can be the result. Another possible mechanism to enhance ROS/RNS influx is an electroporation-like effect of high electrical field originated from plasma. This effect, sometimes called 'plasmaporation', is reported for mammalian cells (see e.g. [235–237]), but is also discussed for microorganism cells [238]. Indeed, a synergistic antibacterial effect of combined treatments of CAP and pulsed electric fields (PEF) was demonstrated [239].

Moreover, products of lipid peroxidation of the membrane of Gram-negative bacteria (*Escherichia coli*) like malondialdehyde are reactive enough to damage DNA and proteins inside the cell.

Inside the cell, several proteins but also DNA are the target of ROS/RNS. In procaryotic cells like bacteria (but not in fungi), DNA is localized in the nucleoid region, but it is not separated by a membrane-bound nucleus from the other cell content. Therefore, DNA might be more susceptible to intracellular ROS/RNS than eukaryotic mammalian cells. The occurrence of ROS/RNS in microorganism cells after plasma treatment is indicated by the upregulation of antioxidative regulatory proteins like OxyR and SoxS in *E. coli*, but also catalases, peroxidases, and SODs. It was also reported that several genes of the SOS response are upregulated, which is a broad regulatory network in most bacteria to counteract DNA damage [160, 240]. Dependent on the intensity (time) of CAP treatment, both DNA damage and the damage to several proteins including enzymes was reported.

Consequently, cell lysis is not the only mechanism of plasma-caused inactivation of microorganisms. There are several physiological and morphological changes of microorganism cells before they are inactivated. More and more studies indicate that also in bacterial cells an apoptosis-like programmed cell death occurs. It can be triggered by the accumulation of intracellular ROS/RNS [241]. This might be one of the potential mechanisms of CAP-induced inactivation of microorganisms.

However, the impact of CAP on bacteria cells may not necessarily result in terminal cell death. External cell stress is able to cause a viable but nonculturable (VBNC) state that is characterized by intact cell membrane, respiratory activity, gene transcription and protein synthesis, but no cell division and, consequently, no colony forming under laboratory conditions [242, 243]. This VBNC state was also reported for CAP-treated bacteria; see e.g. [244, 245]. Because bacteria in this state are not detectable by conventional cultivation methods, this has to be taken into consideration because it could lead to maintenance of contamination or recurrence of infection, respectively.

Particularly important for biomedical applications is the fact that it was proven that a huge number of clinical isolates of wound bacteria, including drug-resistant strains, can be eradicated by CAP. With regard to bacterial resistance to plasma, there are few investigations, only, with divergent results. Here, much more research is needed to clarify if this is a potential point for attention in plasma medicine [246–249].

Summarizing this chapter on CAP effects on microorganisms, it can be stated that CAP has a promising potential to be applied in several fields including plasma medicine. Nevertheless, more intensive and systematic research is needed to explain the detailed mechanisms of inactivation of microorganisms by CAP. One of the central issues is if there is a selectivity of biologically active plasma components between microorganisms and human tissue and what are the reasons or mechanisms of such a selectivity. Approaches to answer

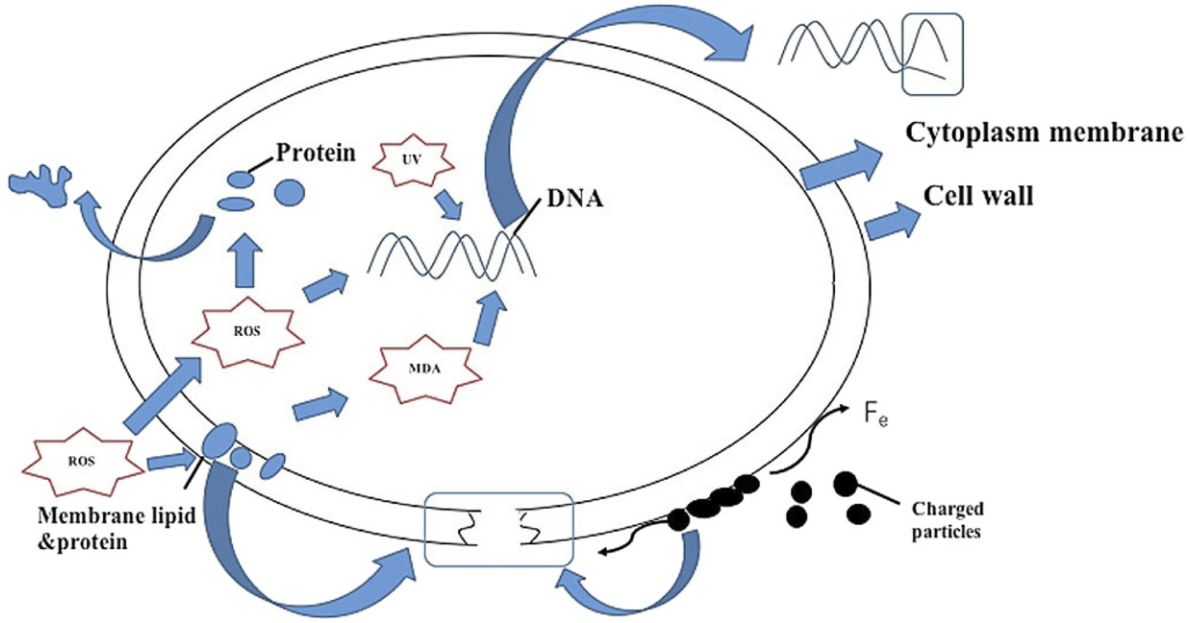

Figure 26. Proposed inactivation mechanisms of microorganisms by CAP; Reprinted from[223], Copyright (2017), with permission from Elsevier.

these questions are required for different cell sizes and structures and different response mechanisms i.e. against oxidative stress. Here, an interesting field of research is still open.

5. Therapeutic applications of cold atmospheric plasma (CAP)

5.1. Wound healing

From the beginning, a central aim of application-oriented research in plasma medicine was CAP application in wound healing [3, 4, 250]. Above all, chronic wounds are an important challenge for patients, health care professionals and health care systems worldwide [251]. Plasma was estimated to have great potential in this field because of its early-predicted effectivity, in two ways: to inactivate wound-contaminating microorganisms and to directly stimulate tissue regeneration [252]. In particular, the latter aspect was extensively researched *in vitro* but also in animal experiments *in vivo* (see section 4). Meanwhile, there are several clinical trials proving the wound-healing effectivity of CAP, summarized in review papers [184, 253, 254].

The first clinical trials were mainly focused on the safe application of CAP devices and the reduction of the microbial load of chronic wounds, see e.g. [7, 255–257]. In these studies, CAP application was used in addition to regular wound care where antibiotic and/or antiseptic therapy was not excluded generally. Moreover, in some studies CAP was tested against local antiseptics. Any statements apllying to wound closure or other indications of healing processes were avoided or mentioned marginally. Consequently, a first meta-analysis considering these studies concluded that the use of CAP 'in wound care is safe, but the retrieved evidence and meta-analysis show that there is no clinical benefit … in chronic open wounds' [258]. This was not surprising because the effectivity of well-proven local antiseptics or systemic antibiotic therapy to reduce the microbial load of chronic wounds is undisputed. Consequently, it was crucial to demonstrate that CAP is more than a 'local physical antiseptic'. Several clinical studies and case reports in human volunteers or patients with well-defined artificial acute wounds without microbial contamination or infection, respectively, could reproduce the results from animal trials, that CAP application is also effective in stimulating tissue regeneration directly, see e.g. [259–261].

Meanwhile, there are meaningful randomized controlled clinical trials available where stimulation of chronic wound healing by CAP was the first objective. In a clinical trial published by Stratmann *et al* 2020 [262], 62 diabetic foot ulcers were CAP-treated over 14 days using the argon-driven plasma jet kINPen MED. A significant reduction of the wound area in the plasma group compared to a placebo group was found (figure 27), whereas both CAP and placebo treatment reduced the bacterial load of the wounds. It has to be noted that all wounds received standard wound care including local disinfection and systemic antibiotic treatment if indicated. Therefore, this study demonstrated beneficial CAP effects on wound healing based on direct stimulation of tissue regeneration independent from antiseptic plasma effects in a randomized, placebo-controlled clinical trial in a population-based, representative cohort of patients.

These results were confirmed in another clinical study including 44 diabetic foot ulcer patients. In this study, a helium-driven high-frequency (HF) plasma jet was used [263].

In a further clinical trial, 37 patients suffering from chronic ulcers of different aetiology were CAP treated one time or three times per week over twelve weeks maximum with the argon-driven plasma torch SteriPlas. Here again, a significant reduction of wound area was found in the CAP-treated groups. Moreover, the CAP treatment three times a week was not

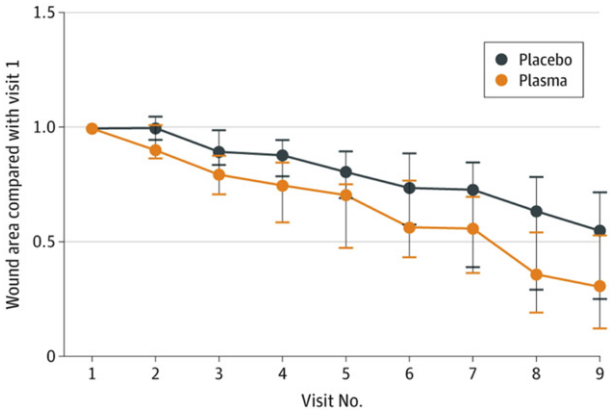

Figure 27. Wound size reduction in 62 diabetic foot ulcers (placebo: 31, plasma: 31) after eight CAP treatments within 14 days (daily treatment for five consecutive days followed by three treatments every second day) in relation to start of therapy; visit nine: two to three days after the last treatment. Reproduced from [262] © 2020 Stratmann B et al. JAMA Network Open. http://creativecommons.org/licenses/by/4.0/

superior to the once-a-week treatment [264]. In a similar study, another argon-driven HF plasma jet treated 50 patients with pressure ulcers once a week over eight consecutive weeks additionally to standard wound care. Both a significantly improved wound healing and a reduction in bacterial load was found [265]. In a case series with 32 patients suffering from venous and mixed leg ulcers, a daily treatment with a needle-type helium-driven plasma device improved wound healing and reduced pain [266].

Consequently, the support of chronic wound healing by CAP is proven according to the current state of clinical research. This is particularly remarkable against the background that generally in the field of wound healing few randomised, controlled trials of wound dressings, bandages etc, which are classified as medical devices, are available because wounds present as part of such a complex presentation that any generalisation within the scope of a clinical trial is estimated to be very difficult [267].

Based on long-term experimental and clinical research, first CAP devices are on the market that are CE-certified as medical devices class IIa. Worth mentioning here are the argon-driven HF plasma jet kINPen MED (neoplas med, Greifswald, Germany), the argon-driven microwave plasma torch SteriPlas (ADTEC, Hunslow, UK), and the DBD-based devices PlasmaDerm (CINOGY, Duderstadt, Germany) and plasma care (terraplasma medical, Garching, Germany). The latter two use atmospheric air as working gas. Currently, these devices are mainly used in reconstitution or re-stimulation of healing processes that are defective caused by other primary reasons resulting in chronic wounds. The direct (initial) stimulation of tissue regeneration is a unique characteristic of CAP with a supportive role of antimicrobial/antiseptic plasma effect. First devices are on the marked since 2013 with increasing successful application in clinical practice. Unfortunately, comprehensible and systematic documentation of this practical experience beyond clinical studies is currently rare.

Reported experimental and clinical results on direct stimulation of tissue regeneration by CAP make it also applicable in the field of acute wound healing, even if acute surgical but also traumatic wounds typically do not need additional stimulation because they are healing regularly under physiological conditions. However, in cases where the risk of retarded healing is enhanced because of the patient's health status, CAP application may be promising, which has been proven in the first case reports [268, 269]. Moreover, the use of CAP for the disinfection of primary and secondary wounds to avoid postoperative wound infection is repeatedly discussed [270]. However, it will be difficult to prove the clinical benefit of such preventive CAP application directly. The same is with the question if any initial acceleration of wound closure by CAP treatment as demonstrated experimentally *in vivo* [263, 271], has any positive impact on scar formation [167]. Nevertheless, together with possible preventive effects on wound infection, CAP treatment is mentioned as a promising option to support several interventions also in the field of plastic surgery and aesthetic medicine [272].

A specific CAP application close to wound healing, which is extremely helpful for the patients concerned, is the treatment of left ventricular assist devices (LVAD) driveline infections. LVAD are mechanical support devices for patients with severe acute and chronic heart failure [273, 274]. These implanted devices are connected to an external power supply by a so-called driveline. The transcutaneous driveline exit site is a conduit for bacterial entrance with the risk of creating a bacterial biofilm growing along the driveline into the body [275]. This can become very dangerous for the patient because the removal and replacement at another site of these driveline catheters is impossible or intricate, respectively. Here, CAP and, above all, the argon-driven jet devices kINPen and SteriPlas demonstrated repeatedly to be very effective tools to fight successfully such driveline infections [276–280].

5.2. Dermatology

Beyond wound healing as the most investigated CAP application, there is some more potential to use plasma devices in a wider context of dermatology [253, 281]. Single case reports, clinical case series and clinical trials are available on CAP treatment of several infective and inflammatory skin diseases with different results.

A randomized placebo-controlled clinical trial is reported including 37 patients suffering from herpes zoster, a viral skin disease characterized by blister formation and painful skin rash in a localized area. Weekday five-minute treatments with the argon-driven microwave plasma torch MicroPlaSter (a predecessor device of SteriPlas) resulted in a more rapid clinical improvement in the first one to two days in the plasma-treated group, including acute pain reduction and initial healing of the herpes zoster lesions [282].

In a randomized two-sided placebo-controlled study with 46 patients with different pruritic (itchy) skin diseases, a 2 min daily treatment with the MicroPlaSter argon plasma torch additional to standard treatment did not result in higher

pruritus reduction compared to placebo treatment with argon gas only [283].

Another relatively widespread skin disease is psoriasis, an autoimmune disease that is noncontagious and characterised by sometimes-large areas of angry, dry, itchy, and scaly skin. In a case series with six patients, a three-times-a-week treatment of psoriatic plaques with the argon-driven atmospheric-pressure plasma jet kINPen showed no significant advantage over conventional therapies. However, because minor positive effects were obtained, it was suggested that it might be worth testing combinations of CAP with conventional topical or systemic therapeutic procedures [284].

Mainly based on the antimicrobial and antiviral effectivity of CAP, single clinical investigations have demonstrated the successful CAP effect on onychomycosis (fungal infection of the nail) and warts [285–287].

Generally, experience of different and more or less successful dermatological applications are mainly anecdotic so far, and are rarely documented or reported in larger case series. However, it is known that medical doctors that are engaged in plasma medicine also try to use CAP in different dermatological indications beyond wound healing.

Very close to therapeutic dermatological applications is the field of plasma applications in cosmetics. It has to be stated that there is not always a clear distinction possible between therapeutic use as it is intended with plasma medicine devices and improvement of appearance, as it has to be intended with cosmetics as well as aesthetic medicine. For instance, CAP application in acne treatment might be focused on both the healing of a skin disease and aesthetic skin improvement [288]. Particularly on the World Wide Web, there are several proposals that offer promising effects of plasma application for corrective treatments and skin improvement, some of them explicitly referring to plasma medicine. Most cosmetic indications of plasma treatment are focused on skin tightening, wrinkle removal, face and body lifting, skin rejuvenation, or blepharoplasty (tightening of eyelids). The techniques are often based on needle-like devices where an electrical spark is generated between the needle-tip and the skin, resulting in dark spots on the skin because of tissue carbonization that resolves during the following days; see e.g. [289, 290], or search the World Wide Web using a keyword like 'plasma cosmetics'. Nevertheless, these applications are often described as 'noninvasive' and 'nonthermal'. The best described and investigated plasma device for cosmetic and aesthetic applications is the nitrogen plasma-based jet-like Portrait PSR^3 system, which is mainly used for skin regeneration. However, its effectivity in acne treatment is also reported. Its activity is based on instantaneous skin heating in a controlled, uniform manner without an explosive effect on tissue or epidermal removal; see e.g. [291–294]. Because most of the known applications of plasma for cosmetic purposes are based on more or less intensive thermal effects, any reference to plasma medicine in terms of this foundation paper, above all, with respect to safety, is illegitimate because nearly all recent investigations into plasma medicine are based on NTP–cell and plasma–tissue interactions. On the other hand, there are several approaches indicating that CAP might also be a promising tool for the stimulation and/or regeneration of skin and, therefore, useful for cosmetic applications. A forward-looking review on the potential of CAP for skin treatments is given in [295]. In particular, the repeatedly reported CAP effect to enhance skin permeability and to enhance the transcutaneous penetration of substances may be promising both for cosmetic purposes and for transcutaneous drug release, but needs much more research, above all, *in vivo*; for more details see e.g. [281, 296–300].

5.3. Cancer treatment

Because of the very promising results in experimental *in vitro* research as well as in animal experiments [9, 12, 22], CAP application in cancer treatment is a current hot research topic in plasma medicine (see section 4.3). However, to realize clinical use of CAP in the field of cancer treatment, some preconditions have to be fulfilled, above all, regarding its applicability and safety. Beside proving CAP selectivity against cancer cells (see section 4.3), another important question surrounds a possible induction of cancer cell growth and proliferation—comparable to the CAP effect in tissue regeneration—with the final consequence of metastasis, if subefficient CAP treatment intensity e.g. in the edge zone of plasma impact, occurs. The first *in vitro* studies give evidence that the latter danger is low [301, 302]. Other *in vivo* studies demonstrated that CAP treatment can enhance tissue oxygenation and modulate blood flow (see section 4.2). On the one hand, this may support metastasis. On the other hand, effects of radiotherapy and chemotherapy may be enhanced in combination treatments. Several synergistic effects of CAP with radiotherapy, PEF, or chemotherapeutics are demonstrated *in vitro* as well as in animal experiments; see e.g. [303–309]. Such combination treatments are under investigation because they offer the chance to reduce effective doses of radiation or chemotherapeutics, respectively, and by this means lower side effects of cancer therapy. Another possible clinical setup is a supportive plasma application to treat the operation field of surgical tumour resections to remove possibly remaining tumour cells in cases where large-scale surgical tumour removal is impossible [310–312]. CAP application in melanoma treatment is another field of long-term research because melanomas typically occur in the skin and are therefore easily locatable and amenable for direct plasma treatment; see e.g. [3, 304, 313, 314]. Due to the potentially induced ICD by plasma (see section 4.3), the melanoma treatment efficacy may be enhanced by combining CAP together with established immunotherapies for metastasized melanoma treatment. Moreover, induction of ICD may open up new perspectives on CAP application for systemic cancer treatment.

However, even if more research has to be done to decide if and under which circumstances therapeutic CAP applications for cancer treatment are promising and realistic in clinical practice, the first clinical applications in specific fields were realized yet. Utilizing especially the antimicrobial plasma effect, CAP application was successful in the palliative care of patients with advanced squamous cell carcinoma of the head and neck, suffering infected tumour ulcerations. As the main intended result, the microbial load and the resulting

typical fetid odour was reduced. Besides additional pain reduction, in some particular cases, transient tumour remission also occurred [17, 315, 316]. However, tumour growth relapsed and the reasons for this effect not enduring in CAP-supported tumour reduction needs to be found [317].

In the treatment of actinic keratosis, a precancerous skin disease which can develop if left untreated, in skin cancer called squamous cell carcinoma, CAP achieved good results in the first patient studies [318–321].

The state of knowledge on CAP effects on cancer cells, including immunostimulatory effects, together with the first clinical applications, characterizes cancer treatment as the next challenging but promising field of clinical CAP application [322]. Further efforts have to be made to find out if CAP will be able to mark a 'paradigm shift in cancer therapy' as was predicted in 2011 [8].

5.4. Other prospective fields

Most plasma devices currently applied in clinical settings or under research for medical applications, respectively, are most applicable on readily accessible places in the human body, e.g. on skin or during open surgery. Another accessible place is the eye. Even though a gentle disinfection of eye surfaces by CAP was demonstrated several years ago [323], plasma application in ophthalmology is a subject of little research so far. It has been repeatedly demonstrated in some studies *in vitro* and in first animal experiments, that CAP is effective to inactivate microorganisms without harmful effects on the cornea [324–328]. In a case series including four patients with therapy-resistant corneal ulcers, the clinical potential of CAP to treat infections as well as ulcerations of the cornea was demonstrated [329].

Another field of long-term research on CAP application is oral medicine and dentistry. Here, several fields of application are thinkable, ranging from implantology, treatment of perimplantitis, treatment of teeth and tooth root channels, treatment of oral wounds and infections up to rather aesthetic applications such as tooth whitening; see for review [330–338]. Over the last 10–15 years there has been a growing number of promising research results being documented. However, CAP applications in clinical settings are rarely reported in the field of oral medicine and dentistry. Obviously, CAP applications have to demonstrate their benefit compared to the more or less satisfactory established therapies in specific applications. Because in most cases dental problems or diseases are not as wearing or life-threatening as nonhealing chronic wounds or cancers, the risk-benefit balance may be assessed in dentistry with a different emphasis compared to other medical fields. Consequently, the realistic potential of CAP application in oral medicine and dentistry has to be evaluated also from an economic point of view [339].

Finally, an intensively researched field hat is not a direct CAP application in medicine is the use of plasma-treated liquids, frequently called PTS, plasma-conditioned liquids, PAL, plasma-activated water (PAW), or plasma-activated medium (PAM). Plasma-induced chemistry was explained in detail in section 3. Liquid phases do not only serve as a transient transport medium to bring ROS/RNS from the plasma/gas phase to the biological target (see section 4.2), but plasma treatment of liquids also results in the enrichment of new chemical species and subsequent changes of their biological characteristics, making them useful for several applications. Most research with respect to medical application of plasma-treated liquids has been done in cancer treatment. Here, two application strategies are followed: (i) direct injection into bulk tumours, and (ii) injection/infusion into the peritoneal cavity in the case of metastasized intraperitoneal tumours, so-called carcinomatosis. For more details, see review papers like [93, 95, 214]. As with direct CAP application for cancer treatment, much more research is needed to bring plasma-treated liquids into clinical therapy concepts. This is with regard to safety aspects on the one hand [340]. On the other hand, the classification of such plasma-treated liquids from a regulatory point of view is currently an open question, which possibly needs new and innovative ways of admission.

6. Perspectives on plasmas for medical applications

Over the years, our understanding of plasma medical applications has continuously increased thanks to the constant efforts of an ever-growing scientific community. Years of multidisciplinary research have laid out solid scientific foundations which have recently started to pay off in terms of development and impact of the technology, as demonstrated by the licensing and commercialization of several plasma sources as medical devices (see section 5.1). After covering these foundational aspects in the previous sections, here we want to highlight what, in our opinion, is still unexplored and pivotal to consider for further developing plasma technology for medical applications. We identified four major challenges: (1) the regulatory challenge, dealing with licensing of plasmas as medical devices apt for clinical use; (2) the cavity challenge, dealing with the development of plasma technologies suitable for treatments in internal cavities of the human body; (3) the dose challenge, dealing with the definition of a concept of dose for medical applications of plasmas; (4) the monitoring and control challenge, dealing with ensuring controlled and repeatable plasma treatments in clinical settings. These challenges often overlap with each other, as in the case of the lack of specific regulations for plasma devices for the treatment of internal cavities of the body. For the sake of clarity, we decided to treat these topics separately in the following, pointing out the connections where needed.

6.1. The regulatory challenge

Regulations of medical devices are intended to guarantee their safety and are rooted in the analysis of the risks associated with the technology under consideration. At the early stage of development of plasma technologies for medical applications, the need to take into account safety aspects was already well understood [3]. The awareness of these aspects grew over the years, with an increasing number of papers referring to the need to consider the compliancy with

regulations in the development of plasma devices [341–343] and citing several documents for the analysis of associated risks [344, 345]. The matter was complicated by the local nature of regulations, often different from country to country, and the exponential increase of activity in the field, resulting in many different plasma sources intended for a plethora of different medical applications. In this context, the year 2014 saw the introduction of the DIN SPEC 91315 [346], a specification registered by the German Institute of Standardization (DIN) and indicating the criteria for the characterization of different plasma devices intended for medical applications. The specification was meant to complement the safety evaluation requirements indicated in the DIN EN 60601-1 [345] by introducing standards for the characterization of plasma sources in order to (i) obtain systematic and comparable results from researchers all over the world and (ii) produce results that could be checked against the safety limits imposed by regulations not specifically developed for plasma technology and that could vary from country to country [344, 347, 348]. The test procedures proposed in the DIN SPEC 91315, in accordance with already available standards [349–354], encompass the measurements of temperature, UV irradiance, emitted gas species, chemical species in liquids, leakage current, antimicrobial activity and cytotoxicity. This specification has been proven as a useful stepping stone for the regulatory approval of plasma sources as medical devices and is, overall, an accepted instrument with several publications reporting the characterization of plasma devices according to its standard procedures [355–357]. Despite all these positive aspects, this specification is rooted in risk analysis performed for plasma devices intended for dermatological applications [358, 359]; as a consequence, the indicated methodologies do not specifically address plasma devices intended for intrabody medical procedures. It should nonetheless be noted that, considered its evolutive nature, the DIN SPEC 91315 could be adapted in the future to account for specificities of intrabody plasma delivery and the associated clinical applications. Also, the DIN SPEC 91315 does not cover the regulatory aspects of plasma-treated liquids. This rarely mentioned aspect [339, 360] is particularly thorny since plasma-treated liquids intended for indirect medical applications can arguably be considered as pharmaceutical compounds, which are associated with harsh regulations, strict manufacturing procedures and the need to univocally identify active principles. It is the opinion of the authors that a discussion on this matter, first among the scientific community and then involving regulatory bodies, is urgent.

6.2. The cavity challenge

Several prospective medical applications of plasmas, chief among which are oncological applications [361], envision intrabody plasma delivery. Plasma devices intended for this scope face peculiar constraints. Beside the requirement of biological efficacy, which is a challenge in itself given the variability of conditions at the site of application, plasma devices have to be flexible and composed of materials with a limited rate of erosion when contacting the plasma. Other aspects that require even more attention for the case of intrabody plasma application with respect to cutaneous application are electromagnetic compatibility, leakage currents, gas delivery and plasma stability. While these constraints have been faced before and were overcome by the plasma community during the development of endoscopic plasma coagulators and ablators [362–366], the nondestructive nature of medical applications of CAP creates an unprecedented challenge. The endoscopic application of CAP was first reviewed by Robert *et al* [367], who classified the different configurations described in literature as capillary DBD, hollow core fiber plasma jets and plasma guns [219, 368–370]. Of these configurations, the latter was arguably the subject of the most studies regarding its characterization and its effects in both *in vitro* and *in vivo* settings [10, 203, 371, 372], confirming the possibility of creating flexible plasma devices capable of exerting biological effects in intrabody settings. More recently, requirements such us material erosion, leakage currents and gas delivery started to be the target of investigation for a new endoscopic plasma source, whose architecture shows several similarities with thermal plasma endoscopic coagulators [373, 374]. Major outcomes of these works are the need for an electronegative shielding gas to prevent the jet-to-glow transition of the discharge, strategies to prevent the formation of parasitic discharges within the plasma device and at the high voltage connections, as well as to limit the erosion of the materials during operation. Despite these advancements, to the best of our knowledge there are currently no endoscopic CAP sources licensed as medical devices. Following the footsteps of plasma devices for dermatological applications, the road towards preclinical and clinical studies will require the production of an ample body of documentation regarding the characterization, the safety and the biological efficacy of endoscopic plasma sources. A standardization of procedures, adapting and extending the DIN SPEC 91315, would prove instrumental to reach this goal.

6.3. The dose challenge

The term ‘dose’ refers to the quantity of a therapeutic agent or of radiation, in the case of radiotherapy, administered to the patient during a treatment. Medical doctors rely on this concept when considering the therapeutic window of a certain drug/treatment, whose boundaries are defined by the minimum quantity required to exert the desired biological effects (minimum effective dose) and the maximum quantity not eliciting unacceptable toxic effects (maximum tolerated dose). Moreover, the dose offers a standard to compare different treatments and is a suitable control parameter, since it connects the inputs (the administered quantity of therapeutic agent) with the outputs (the biological effects) of the process. In the context of plasma medical applications, dose initially referred to the interrelated parameters of treatment time, power coupled with the discharge and energy dissipated in it [152, 375–382]. As the understanding of the fundamental mechanisms driving plasma medical applications progressed over the years, the dose became a controversial concept, with increasing discussions on the limitations of an energy-related definition [111, 184]. A more refined concept of dose was recently

proposed. It relies on the equivalent total oxidation potential (ETOP) and the bacterial reduction factor [133]. The ETOP accounts for the oxidation potential of (i) the reactive species produced in the discharge and flowing towards the target; (ii) the other agents associated with the plasma discharge, such as UV radiation and electric fields; and (iii) the synergistic effects of the previous two factors, and was shown to be a promising instrument to predict the bactericidal potential of some plasma sources. Nonetheless, a comprehensive description of dose through the ETOP parameters faces many obstacles. First of all, determining the oxidation potential of the reactive species requires the measurement of their flow towards the substrate in real time, posing significant challenges in terms of process monitoring. Second, quantitatively determining the oxidation potential of agents other than reactive species is complicated. Third, determining the oxidation potential associated with the synergistic effects would require a complete mechanistic comprehension of the plasma biomedical processes. Furthermore, the proposed parameter does not account for the oxidation potential of the reactive species produced in the liquid environment surrounding the tissues and has yet to be shown to be suitable for therapeutical applications different from disinfection. As a final note, data-driven machine learning techniques have recently been proposed as useful means to support in the identification of a suitable parameter to be employed as the dose for plasma medical applications, or as a possible strategy to altogether circumvent the need for a definition of dose [383].

6.4. The monitoring and control challenge

Clinical applications of plasmas require repeatable and safe treatments, often creating the need for refined process control strategies. More specifically, the typically nonlinear relation between the process inputs (plasma parameters) and outputs (the biological effects) can be tackled with feedback control strategies. This means the creation of a loop where process inputs are adapted as a function of the process outputs; an example of this strategy has been previously reported for the case of a plasma jet and a simplified set of process parameters [384]. Controlling strategies, in principle, require an understanding of (i) which are the relevant parameters controlling the process; (ii) what is the relationship between inputs and outputs of the process; and (iii) monitoring those inputs and outputs. In the case of plasma technologies, each of these points is a challenge in itself, since the process presents multiple variables (active principles) with synergistic effects and the understanding of the fundamental mechanisms driving plasma-induced biological effects is still incomplete. Last but not least, real-time monitoring of plasma devices is often impossible in a clinical setting with conventional diagnostic techniques, either due to their nature, as in the case of LIF or mass spectroscopy for measuring gas phase reactive species, or due to the computational time required to elaborate the data acquired with more suitable techniques, such as OES. Stimulated by the increasing use of machine learning strategies in medicine [385–388], artificial intelligence data-driven approaches have been proposed as a potential solution for these issues, and also in the field of plasma medical applications [383, 389]. These techniques seem to offer interesting avenues for identifying correlations between input and output parameters, for highlighting patterns among large amounts of data-produced monitoring plasmas and for enabling suitable feedback control strategies [390]. Only the first steps have been taken on this path and the road ahead is littered with scientific questions and nontrivial technological challenges.

7. Summary

Plasma medicine, i.e. the use of cold atmospheric-pressure plasma (CAP) directly on or in the human or animal body for therapeutic purposes, has made tremendous progress over the last decade. Even if a large number of different CAP devices is in experimental or clinical application, two basic types are dominating: DBD and APPJs. A common feature of all CAP devices is that they are working under ambient air conditions or, in several cases, using ambient air as the working gas for plasma generation. This results in the generation of reactive oxygen and nitrogen species (ROS, RNS) which were identified as the most important plasma agents causing specific biological effects of CAP. The composition and quantity of ROS/RNS in plasmas depends on the diverse physical and technical parameters of the plasma-generating devices as well as on ambient conditions and plasma contact with other media beyond atmospheric air, above all, liquid phases. Therefore, the biological performance of CAP devices may vary in detail. However, plasma-generated ROS/RNS are similar to those occurring as part of the metabolism in living organisms and play several roles in cellular physiology and biochemistry. Therefore, redox biology serves as a scientific basis to decipher and understand molecular mechanisms of biological CAP effects and its medical use. Moreover, because of the physiological occurrence of ROS/RNS, mammalians (as humans are) in particular are able to control its concentration in cells and tissue and largely prevent any harmful effects of these species. Above all, mutagenic and genotoxic effects can be excluded if CAP devices are applied adequately.

CAP application in wound healing is on its way into clinical routine. The first CAP devices are approved for this field of medical application. Mainly in chronic wounds, the unique combination of the broad-spectrum antimicrobial effectivity of CAP with a direct stimulation of tissue regeneration contributes to physiological wound healing that may be disturbed by other diseases. Another important feature of CAP is its ability to induce regulated cell death in mammalian cells dependent on CAP treatment intensity. This is also true for cancer cells. Even if CAP application in cancer treatment is still currently at the preclinical research stage, it is to be expected that it will be the next step in clinical plasma application.

Today it has to be stated that the medical application of CAP, which was primarily facilitated by physicists, has become a transdisciplinary research field combining expertise from plasma physics, life sciences, and medicine. This close cooperation is the key to ongoing successful research and development leading not only to new options in medical

therapy but also to a sound scientific basis for the innovative field of plasma medicine.

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Data availability statement

No new data were created or analysed in this study.

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