Development of a safe therapeutic window for cold atmospheric plasma treatments

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1. Summary

According to the WHO surveillance report in 2014 antimicrobial resistance is an increasingly serious threat to global public health. Infections are becoming harder or impossible to control; the risk of infections spreading to others is increased; illness and hospital stays are prolonged, with added economic and social costs. The problem is so serious that it threatens the achievements of modern medicine.

Lately the use of cold atmospheric plasma (CAP), a partly ionized gas at room temperature and pressure, has developed into an innovative field in medicine and hygiene. Due its antimicrobial properties cold atmospheric plasma aroused attention in dermatology; possible application fields are wound disinfection and the treatment of other pathogen-caused skin diseases. However, this technology is rather novel and the medical and hygiene community has to be convinced that CAPs are safe, controllable and efficient. The motivation of this study was therefore to find a “therapeutic window” where bacteria are inactivated and human skin is not damaged.

For this purpose, the bactericidal efficacy of CAP in this work was examined in more true-to-life conditions than previous studies. The bactericidal effect of CAP was examined in solution, as the wound environment is rather wet. High reduction rates of *E.coli* of more than 5 log steps were achieved in less than 3 minutes of CAP application.

Besides achieving high bacterial reductions it is further important to investigate patient safety i.e. if CAP can be applied to human skin or other eukaryotic cells in general. By carrying out the hypoxanthine-guanine phosphoribosyl transferase

(HPRT) assay the mutagenic potential of the used CAP source was therefore investigated.

CAP produced by the used device did not evoke mutations beyond naturally occurring spontaneous mutations. In addition to that, the impact of CAP treatment on the cell biology of primary human dermal fibroblasts including proliferation, cell cycle distribution and migration was examined. The results show that CAP application only slightly inhibited cell proliferation and migration and that a cell cycle arrest was only detectable for high dosages.

The data of this work clearly reveal CAP as a promising candidate for efficient and safe wound disinfection. This paves the way for new medical treatments and the possibility in future to use designed plasma in several application fields.

2. Zusammenfassung

Laut des Überwachungsberichts der WHO im Jahr 2014 ist Antibiotikaresistenz eine zunehmend ernste Bedrohung für die globale öffentliche Gesundheit. Es wird immer schwieriger bis unmöglich Infektionen zu kontrollieren; das Risiko einer Ausbreitung von Infektionen auf andere ist erhöht; zusätzlich zu wirtschaftlichen und sozialen Kosten verlängert sich die Dauer von Krankheiten und Krankenhausaufenthalten. Das Problem ist so gravierend, dass all die Errungenschaften der modernen Medizin bedroht sind.

In den letzten Jahren hat sich die Verwendung von kaltem Plasma bei Atmosphärendruck (CAP), einem teilweise ionisierten Gas, zu einem innovativen Bereich der Medizin und Hygiene entwickelt. Aufgrund der bakteriziden Wirkung von CAP erregte es Aufmerksamkeit in der Dermatologie; mögliche Einsatzgebiete sind Wunddesinfektion und die Behandlung von anderen, von Pathogenen hervorgerufenen Hautkrankheiten. Diese Technologie ist jedoch eher neu und muss daher erst die Medizin- und Hygienegemeinschaft von seiner Sicherheit, Kontrollierbarkeit und Effizienz überzeugen. Die Motivation dieser Arbeit war deshalb, ein therapeutisches Fenster zu finden, in dem Bakterien abgetötet werden und menschliche Haut nicht geschädigt wird.

Zu diesem Zweck wurde in dieser Arbeit die bakterizide Wirksamkeit unter Bedingungen untersucht, die realitätsnaher waren als die in vorherigen Studien. Der antibakterielle Effekt wurde in Lösung untersucht, da Wunden auch eine nasse

Umgebung darstellen. Hohe Reduktionsraten von *E.coli* von mehr als 5 log Stufen konnten in weniger als 3 Minuten Plasmabehandlung erzielt werden.

Genauso wichtig wie hohe Reduktionsraten ist die Untersuchung der Patientensicherheit, d.h. ob CAP auf menschlicher Haut oder generell auf eukaryotischen Zellen angewendet werden kann. Mit der Durchführung des Hypoxanthin-guanin Phosphoribosyl Transferase (HPRT) Tests wurde das deswegen mutagene Potential von CAP untersucht. Das von dem hier genutzten Gerät produzierte Plasma rief keine Mutationen zusätzlich zu den spontanen, natürlich auftretenden Mutationen hervor.

Zusätzlich dazu wurde der Einfluss von CAP auf die Zellbiologie von menschlichen, primären Haut Fibroblasten hinsichtlich Proliferation, Zellzyklusverteilung und Migration untersucht. Die Ergebnisse zeigen nur eine geringfügig verminderte Zellproliferation und –migration und dass der Zellzyklusarrest nur für hohe Dosen nachweisbar war.

Die Daten dieser Arbeit weisen deutlich darauf hin, dass CAP ein vielversprechender Kandidat für effiziente und sichere Wunddesinfektion ist. Es ebnet den Weg für neue medizinische Behandlungsmethoden und die Möglichkeit, designtes Plasma zukünftig in weiteren Anwendungsbereichen zu verwenden.

3. Introduction

As history shows, new tools are often found by accident. Since 99% of the visible universe consists of plasma, all its forms are common objects of investigation in space research. While hot plasma has been used for years to sterilize surgical instruments it is limited to thermo-stable materials and environments. After it was possible to generate plasma at atmospheric pressure and under ambient conditions, plasma physicists like Prof. Laroussi had the idea to examine the effects of cold atmospheric plasma (CAP) on living bacteria. [1] They found that CAP has very strong antibacterial effects. In 2005 the research field “plasma medicine” was born when the group of Prof. Morfill at the MPE started the first clinical study worldwide with CAP on human wounds. Cold atmospheric plasma (CAP) is an upcoming technology due to its broad antibacterial properties, [2-10] the probable lack of resistance against it [11, 12] and the potential to preserve the functionality of the surrounding human cells. [13-16]

3.1 Plasma

In medicine plasma normally describes blood plasma. But plasma in this context is the so called fourth state of matter. Sir William Crookes, an English physicist, first described it in 1879. In 1929, the term “plasma” was first applied to ionized gas by Dr. Irving Langmuir, an American chemist and physicist [17]. “Ionized” means that at least one electron is not bound to an atom or molecule, converting the atoms or molecules into positively charged ions. As temperature increases, molecules become

more energetic and the state changes from solid over liquid to gaseous and finally to plasma, which justifies the title “fourth state of matter”. [18]

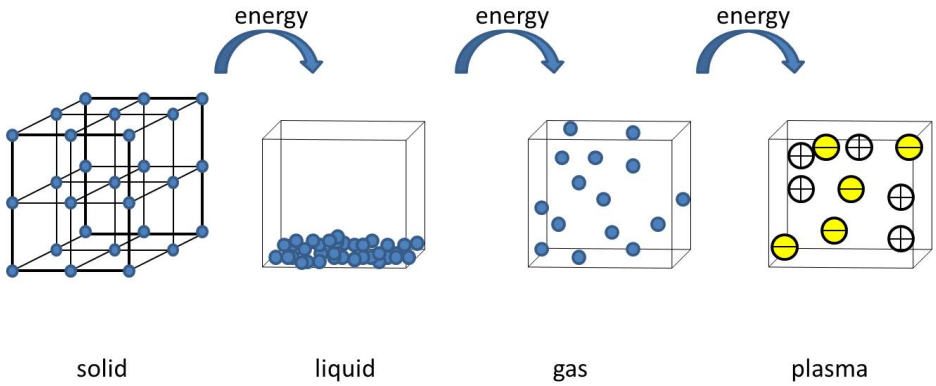


Figure 1: The four states of matter. By addition of energy matter is transformed from solid over liquid and gas to plasma.

The free electric charges – electrons and ions – make plasma electrically conductive, internally interactive, and strongly responsive to electromagnetic fields. An ionized gas - both natural and artificially generated - is usually called plasma when it contains well balanced numbers of negative and positive charged particles, which means quasi-neutral. Neutral particles may be present as well. The relative number of ions and atoms (the ratio of density of major charged species to that of neutral gas, $\frac{n_i}{n_a}$) is called the degree of ionization. The ionization energy, or ionization potential, is typically specified in electron volts (eV) and refers to the energy required to remove a single electron from a single atom or molecule. [18]

High-temperature plasma is an established method to sterilize equipment or to remove/destruct, cut and cauterize tissue and most recently for cosmetic reconstruction of tissue. In the past years, researchers found out, that, due to reactive species, charging reactions, ultraviolet radiation (UVR), optical and infrared

emissions and heat, plasma has bactericidal and fungicidal effects. [19, 20] It depicts a practicable tool in application areas where temperature is not an issue. But when it comes to living tissues and thermo labile materials temperature is a limiting factor. And above all, vacuum chambers are not practicable in clinics or everyday life. The challenge was therefore to bring the plasma, with all its benefits, down to room temperature and atmospheric pressure.

3.2 Cold atmosphere pressure plasma (CAP)

In the last two decades research has succeeded in creating cold plasma at atmospheric pressure. Cold atmosphere pressure plasma (CAP) has similar benefits as high-temperature plasma with all its characteristics, but without the enormous heat production.

Usually very high temperatures and high energy input are needed for ionization. The electron temperature is relatively high; therefore plasma is referred to as being "hot”. Sometimes the term "cold plasma" is used when the temperatures of the present ions and neutrals are much lower than the electron temperature, i. e. if the particles in the plasma are not in a thermal equilibrium. Numerous plasmas exist far from the thermodynamic equilibrium, and are characterized by multiple temperatures, related to different plasma particles and diverse degrees of freedom. It is the electron temperature T_e that often significantly exceeds the temperature of heavy particles T_0 ($T_e >> T_0$). Ionization and chemical processes in such nonequilibrium plasmas are mainly determined by the electron distribution function, therefore are not sensitive to thermal processes and temperature of the gas. Plasma in a nonequilibrium state is usually called nonthermal plasma. An example of nonthermal plasma in nature is the aurora borealis.

In many nonthermal plasma systems, electron temperature is in order of several eV (1 eV approximately equals to 11,600 K), whereas the temperature of neutrals and ions is close to room temperature. Nonthermal plasmas are usually generated either at low pressures or at lower power levels compared to thermal plasmas, or in different kinds of pulsed discharge systems.

Emission of light and heating are by-products of the plasma generation process. The rates of such processes strongly depend on power input and the used gas composition and can often be far from the equilibrium distribution. Chemically active plasmas exhibit multi-component systems that are highly reactive due to large concentrations of charged particles (electrons, negative and positive ions), excited atoms and molecules, reactive species, and UV photons.

Ions are heavy particles, so usually they cannot receive high energy directly from electric fields due to intensive collisional energy exchange with other plasma components. These collisions with plasma components are the principal process for the formation of reactive species. [18]

There are different ways to create cold plasma at atmospheric pressure. Plasma ignition can be pulsed. This means that the ambient air is only partially ionized and electrons are energized whereas at the same time the ion temperature remains at room temperature. Another possibility of reducing the temperature of the plasma system (but not of the electrons) would be to dilute the “hot” plasma components by inducing a gas flow.

In general CAPs can be classified into three types: direct plasmas, indirect plasmas and “hybrid” plasmas (Table 1).

Table 1: Characteristics of different CAP technologies. [12]

	Direct plasmas	Indirect plasmas	"Hybrid" plasmas
plasma sources / examples	Floating electrode dielectric barrier discharge (FE-DBD)	Plasma jet, plasma plume, plasma bullets, plasma torch (e.g. MicroPlaSter), plasma gun	Surface-Micro Discharge (SMD)
gas	air	noble gas / air	air
Current through the treated sample	high	low	low
UV radiation	relatively weak	relatively strong	relatively low
Reactive species	are produced in the plasma	are produced by mixing plasma and air	are produced in the plasma
plasma species which reach the treated sample	Reactive species and positive/negative ions	Mainly reactive species	Mainly reactive species and a small amount of positive/negative ions

Table 2 gives a small overview of research groups, which are working with cold atmosphere pressure plasma, including the main technology they use.

Table 2: Examples of groups all over the world which are working with different cold atmospheric plasma sources. The table raises no claim to completeness.

Group	Technology
Drexel University, Philadelphia, USA	FE-DBD [21]
Gamaleya Research Institute, Moscow, Russia	MicroPlaSter ß, argon flow [22]
George Washington University, Washington D.C., USA	Plasma jet, helium flow [23]
GREMI, University of Orlèans, France	DBD in air [24]
HuaZhong University, China	DBD plasma plum, helium flow [25]
INP Greifswald, ZIKplasmatis, Germany	DBD plasma jet (kinPen) [26]
Loughborough University, Great Britain	Microplasma jet, helium flow [3]
Max Planck-Institute, Garching, Germany	MicroPlaSter ß (argon flow) [13], SMD in air [27]
Old Dominion University, Norfolk, USA	plasma pencil, bullets, helium flow [28]
Pohang University of Science and Technology, Republic of Korea	Pulsed plasma jet [29]
University of California, Berkeley, USA	SMD in air [4]
University of Eindhoven, Eindhoven, Netherlands	RF-DBD, plasma needle, argon/oxygen mix flow [30]
University of Nagoya, Nagoya, Japan	DBD, argon flow [31]

Direct plasmas

Devices with floating electrode dielectric barrier discharges (FE-DBDs) work with the (biological) sample as a counter electrode and are therefore called direct plasmas. FE-DBDs produce the plasma in the gap between the living tissue and the insulated electrode of the device thereby generating high concentrations of plasma species (Figure 2).

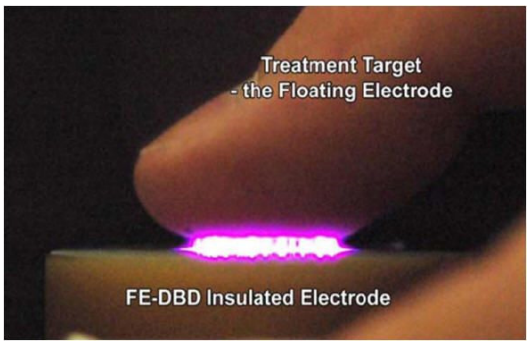


Figure 2: Floating Electrode Dielectric Barrier Discharge (FE-DBD) device. The plasma is produced between the insulated electrode of the device and the treatment target (finger) – the floating electrode. The picture was taken from Fridman et al. [18]

Indirect plasmas

A very popular way to create cold plasma is called indirect. Here plasma is produced between two electrodes and then transported to the sample via a gas flow. The transport of the charge carriers (and the produced molecules) away from this discharge region results from the gas flow and diffusion. Most current CAP plasmas are generated in helium or argon mixed with a small amount of reactive gases such as oxygen; however, they can also be generated in molecular gases such as air. [32, 33] As air is mainly composed of nitrogen (approx. 78%) and oxygen (approx. 21%) the generation of cold atmosphere pressure plasma in air mainly results in formation

of reactive nitrogen and oxygen species. [34-37] Several indirect plasmas have been developed - one example is the so called plasma jet. [38-42]

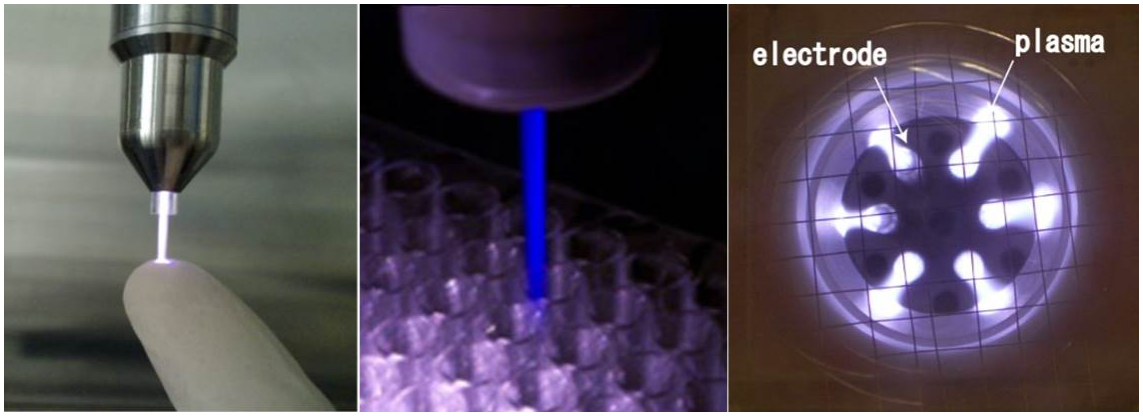


Figure 3: Different representatives of indirect plasma sources- plasma jets [41, 43] and the plasma torch (MicroPlaSter ß).

"Hybrid" plasmas

So called "Hybrid" plasmas represent a combination of direct and indirect plasmas. The plasma is created by electrodes and the produced species are transported mainly via diffusion onto objects. There is no external gas flow applied. This technique is the basis for the so called Surface Micro - Discharge (SMD) technology and is described in detail in the next chapter as it is the basic technology of all devices that were used in this study.

3.3 SMD Device

The Surface Micro - Discharge (SMD) technology formed the foundation to build low- price, robust and scalable plasma electrodes. [44] The SMD devices used in this study are producing cold, non-thermal plasma at atmospheric pressure by short intervals (in the range of 100 ns) of plasma ignition resulting in only partial ionization of the ambient air. Thereby, electrons are energized and reach temperatures of a few eV while simultaneously the ion temperature remains at room temperature. The overall temperature of the system does not exceed room temperature since the ionization degree is low - at its maximum 10^{-7}.

The basic principle of such a SMD electrode is shown in Figure 4. The SMD electrode is composed of a sheet electrode, an insulator and a grounded mesh grid which are mechanically pressed together. By applying high voltage across the dielectric in the range of kV at frequencies in the range of kHz the structured (mesh grid) electrode creates local “hotspots” in electric field strength, which enables the formation of the microdischarges.

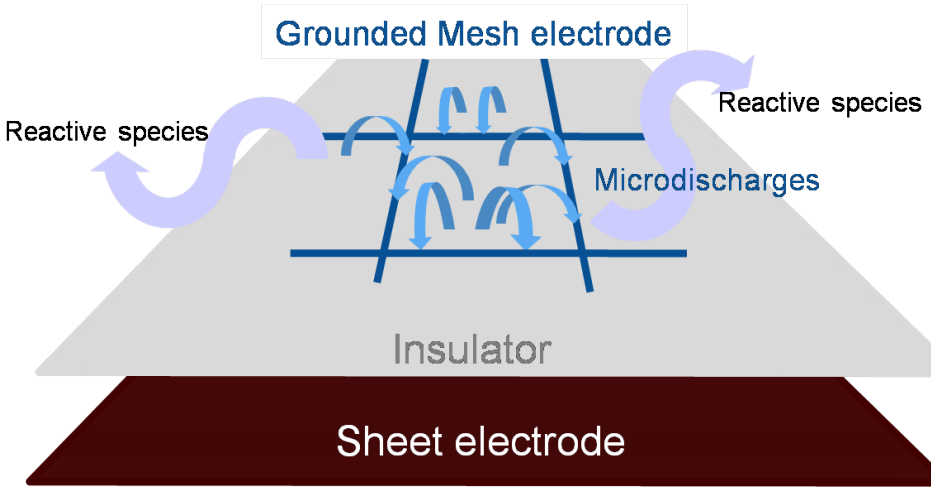


Figure 4: Basic principle of the Surface Micro - Discharge (SMD) technology. [20] On the surface of the mesh grid many (Micro)discharges produce a small layer of plasma.

Based on the SMD technology a device was established to fit conditions in a biological laboratory, the so called FlatPlaSter 2.0. For clinical applications and domestic use, this technology was also built into a small handheld device with higher power consumption, called MiniFlatPlaSter. To be able to apply different power modes on the same electrode a mode transition electrode was finally developed.

Depending on the power input the proportion of the individual species and components varies, but the principal composition is the same for all three devices: reactive oxygen and nitrogen species; electrons; charged particles; UV light; heat.
In a closed volume the temperature during plasma application increases 0.2°C/min. This has neither for prokaryotes nor for eukaryotes a remarkable effect.

Main UV components emitted by the devices are in the wavelength range between 280 and 400 nm and correspond to the N_2 second positive system (Figure 5). Furthermore, insignificant intensities of UVC light emission can be detected. The UV power density was measured to be 25 nW/ cm^2. Altogether this UV light emission has

no biological effect and therefore doesn't need not to be taken into account for this study.

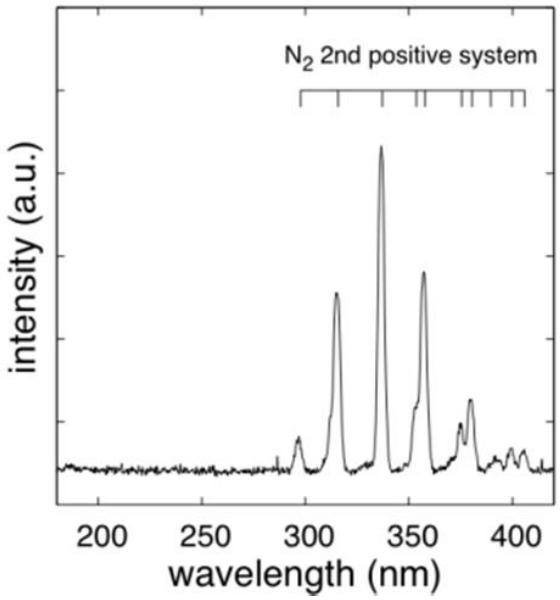


Figure 5: Optical emission spectroscopy in the low power mode in air. No emission of UVC light, only low dose emission of UVB and UVA light could be detected (manuscript in preparation by Shimizu et al.).

The micro-discharges of the electrode have an extent of typically 2 mm and tend to align along the dielectric surface. Hence the plasma forms a very thin layer of less than one mm thickness. Therefore only a small fraction of the plasma itself (electrons and ions) and the short-lived species may reach the treatment sample, which is typically placed a few mm to cm away from the electrode. Simulations using computational modelling contained more than 600 chemical reactions for the analysis of the plasma discharge. But mainly long-lived components such as O_3, NO_2, N_2O, N_2O_5, HNO_2, HNO_3, H_2O_2, etc. reach the treatment area via diffusion and/or convection.

The major reaction pathways in the region under the discharge layer are those with ozone. Figure 6 shows that the measured ozone density varies in SMD plasmas for different power input. For low power input (black curves with power consumptions ranging from 9.5·10-4 W/cm^2 to 0.10 W/cm^2) the ozone concentration increases monotonically (in some cases even up to several 1000 ppm) and finally saturates.

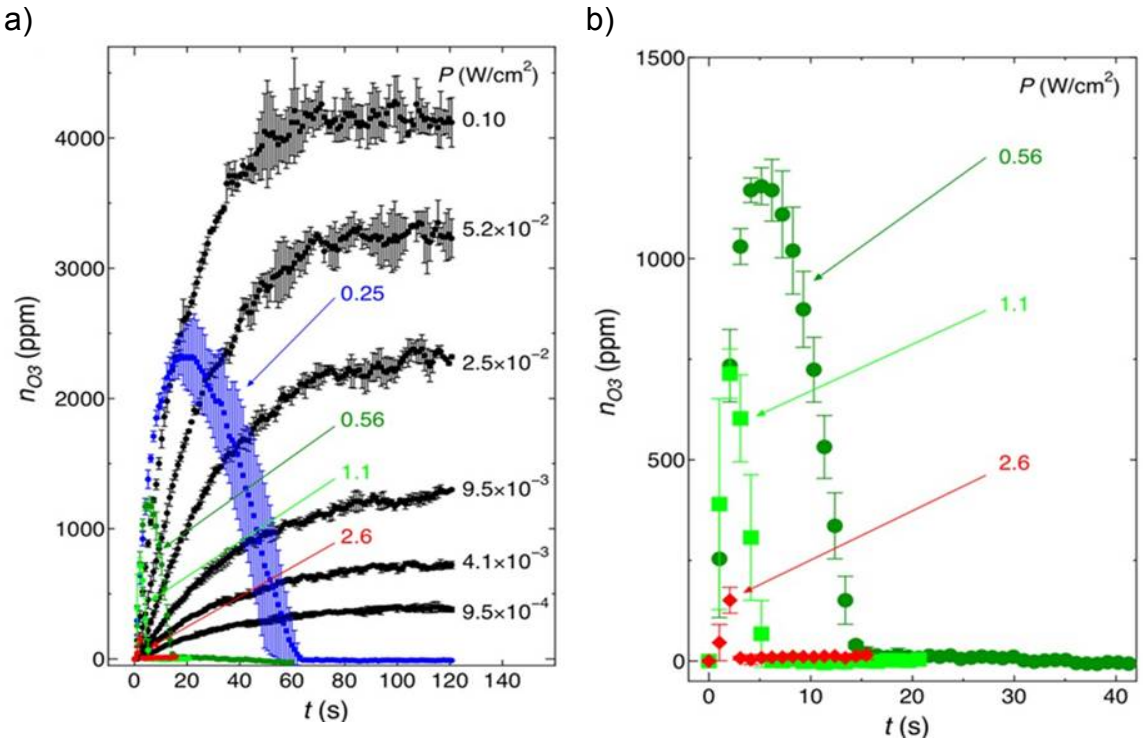


Figure 6: Time dependent ozone production of the SMD device in air.
a) Time evolution of the ozone density (n_{O_3}) produced by the SMD electrode (in a confined volume of 5 cm^3) for different power consumptions measured with UV absorption spectroscopy at 254 nm. The power consumption of the FlatPlaSter 2.0 is approximately 0.02 mW/cm².
b) Time evolution of the ozone density with power consumptions of 0.56 W/cm^2 and higher. The applied voltage was 15 kVpp. The power consumption of the MiniFlatPlaSter is approximately 0.5 W/cm². [35]

For higher power input (green and red curves with power consumptions ranging from 0.25 W/cm^2 to 2.6 W/cm^2) the ozone concentration first increases, but afterwards decreases again immediately. Model calculations suggest that the ozone depletion at higher power densities is caused by quenching reactions with nitrogen oxides that

are in turn created by vibrationally excited nitrogen molecules which react with O atoms:

$$N_2 + O_2 \rightleftharpoons 2\,NO$$

$$2\,NO + O_2 \rightleftharpoons 2\,NO_2$$

$$O_3 + NO \rightarrow NO_2 + O_2$$

$$O_3 + NO_2 \rightarrow NO_3 + O_2$$

It is well known that the ozone emission from the plasma discharge is a key problem for *in vivo* applications if atmospheric plasmas in ambient air are used. The Immediately Dangerous To Life or Health Concentrations (IDLHs) given by the US National Institute for Occupational Safety and Health (NIOSH) for ozone is 0.1 ppm for 8 hours continuous exposure per day (for 6 days a week) (www.cdc.gov/niosh). But as described above when plasma is ignited in a closed volume ozone will be quenched and converted into nitrogen oxides. For NO, the IDLH is 100 ppm and 1 ppm for NO_2.

As the current SMD devices mainly use the surrounding air for plasma production, the produced plasma chemistry will change if the humidity or ambient temperature varies. Humidity ranges from 20-80% and temperature ranges from 15-35°C do not affect the bactericidal properties of CAP, [45] but changing conditions may have an influence on CAP effects on eukaryotic cells and tissue and therefore needs to be controlled carefully. Alteration of the power input leads to a change from a mainly ozone based regime (low power mode) to a nitrogen based regime (high power mode). In a collaboration with the Chemical and Biomolecular Engineering Faculty at the University of Berkeley this ‘mode transition’ was confirmed by Fourier transform

infrared spectroscopy (FTIR) measurements carried out with a SMD electrode with a power input of 0.3 W/cm^2 (Figure 7 a). In addition species such as N_2O, NO_2 and HNO_3 were identified. Interestingly these measurements also revealed that the experiment itself can influence the produced plasma chemistry of the SMD electrode showing that if a thin liquid film is present, e.g. if cells covered with medium are treated with CAP, a reduced decline rate of O_3 can be found (Figure 7 b).

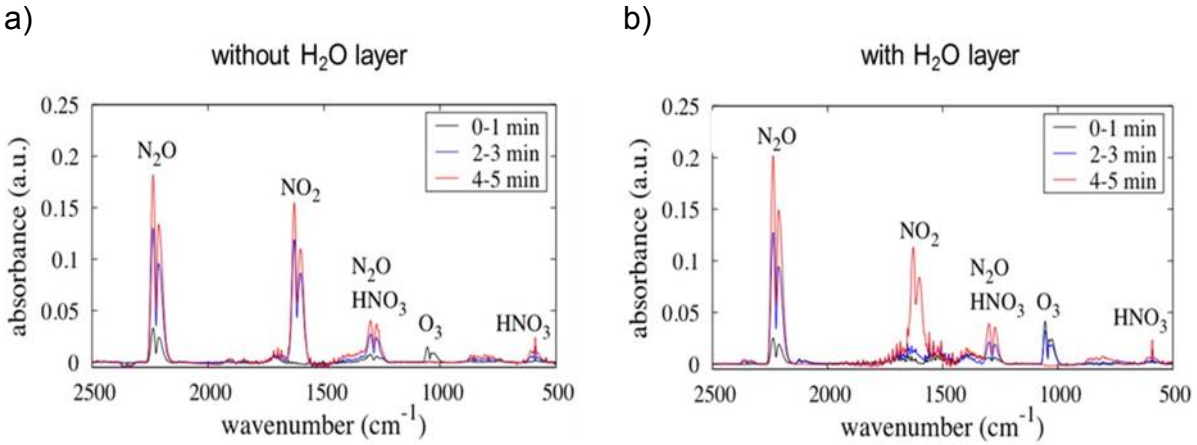


Figure 7:
a.) IR absorption spectrum of the plasma gas (power consumption 0.3 W/cm^2) in the absence of H_2O.
b.) IR absorption spectrum of the plasma gas (power consumption 0.3 W/cm^2) in the presence of a H_2O layer.

3.4 Chemistry in liquids

Plasma is produced on a mesh grid some millimetre above the sample. The biological sample is either in solution or dry, only covered by a thin layer of liquid. All components in the plasma diffuse through a so called “afterglow region” between the electrode and the sample and are then dissolved by the liquid (Figure 8).

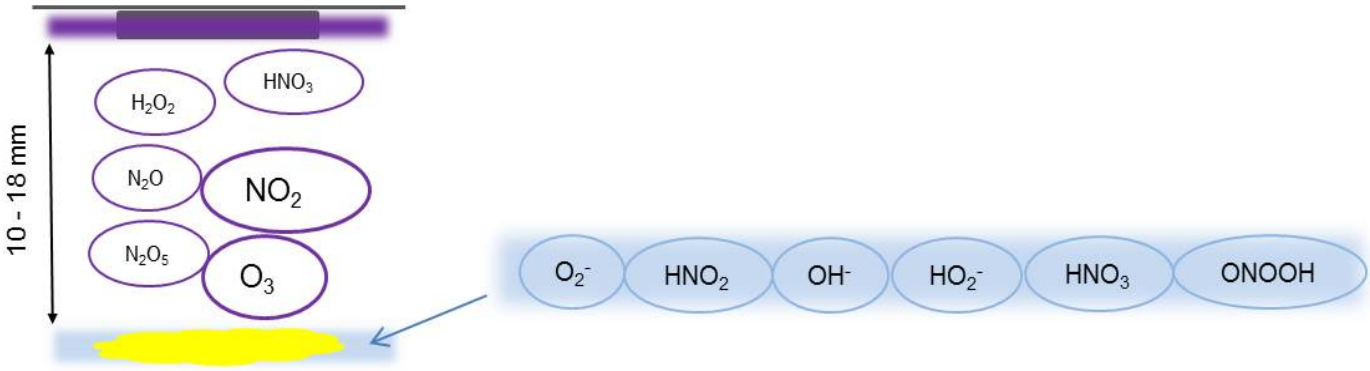


Figure 8: Schematic drawing of species in air and in liquid.

As mentioned before, only long living species such O_3, NO_2, N_2O, N_2O_5, HNO_2, HNO_3, H_2O_2 reach the sample, in which O_3 and NO_2 are quantitatively the most important. [36]

As the sample is covered by a thin layer of liquid, reactive species then dissolve into the liquid. This process underlies the law of Henry:

$$k_H = \frac{c_H}{p_H}$$

where c is the concentration (or molarity) of gas in solution (in mol/L), p is the partial pressure of the gaseous solute H above the solution (in bar) and k_H is a constant with the dimensions of pressure divided by concentration. The constant k_H, known as the Henry's law constant, depends on the solute, the solvent and the temperature.

Table 3 gives the Henry's law constants for the important species of plasma which reach the sample.

Table 3: Henry's law constants of important reactive species involved in the plasma chemistry. [National Institute of standards and technology]

species	Henry's constant [$\frac{mol}{L \cdot bar}$]
O_3	0.011
NO_2	0.012
N_2O	0.025
N_2O_4	1.6
H_2O_2	82000

As an example, the calculation for ozone in the setup for the FlatPlaSter 2.0 with cells (Figure 10) is shown. As the plasma devices are working in ambient air, we use the ambient pressure for our calculations. We calculate with a minimal height of the liquid of 1 µm (cell culture dish r = 5 cm) as the medium is sucked off the sample before the plasma treatment. The distance between the electrode and the sample is 18 mm and the volume is closed. Therefore we have an air volume of 0.000142 m³. From the measurements of the ozone evolution we know, that after 1 minute CAP application the ozone concentration is 550 ppm, which equals to 550 ml/m³. This corresponds to a partial pressure of O_3 in air:

$$p_{O_3} = 0.56\ mbar.$$

With this we can calculate the concentration of ozone in solution:

$$c_{O_3} = k_{O_3} \cdot p_{O_3} = 0.0011\ \frac{mol}{L \cdot bar} \cdot 0.00056\ bar$$

$$c_{O_3} = 62\ \mu M.$$

In our setup with a liquid volume of 8 µl this results in 0.0005 µmol solved ozone (corresponding 0.024 µg).

In aqueous solutions ozone decomposes mainly to O_2, HO_2 and OH. The dissolved ozone initiates a reaction with the hydroxyl radical (OH-), resulting in the production of HO_2 and the superoxide radical (O_2^-). [46]

$$O_3^- + H_2O \rightarrow OH + O_2 + OH^-$$

$$O_3^- + OH \rightarrow O_2^- + HO_2$$

$$O_3 + OH^- \rightarrow HO_2^- + O_2$$

Furthermore, O_2^- is converted to the hydroxyl radical (OH^-) and nitrogen dioxide (NO_2) in the presence of the nitrosyl cation (NO^+). [47-51]

$$O_2^- + NO^+ + H_2O \rightarrow 2OH^- + NO_2$$

$$NO^+ + H_2O \rightarrow HNO_2 + H^+$$

$$4NO_2^{\cdot} + O_2 + 2H_2O \rightleftharpoons 4HNO_3 \rightleftharpoons 4ONOOH$$

Pavlovich and colleagues measured the key chemical species in CAP treated aqueous buffer (PBS) using a SMD device similar to the FlatPlaSter 2.0. [52] The results revealed that the aqueous chemistry corresponds to the air plasma chemistry – i.e. a transition from the ozone mode to the nitrogen mode can be observed as the discharge power density increases (Figure 9).

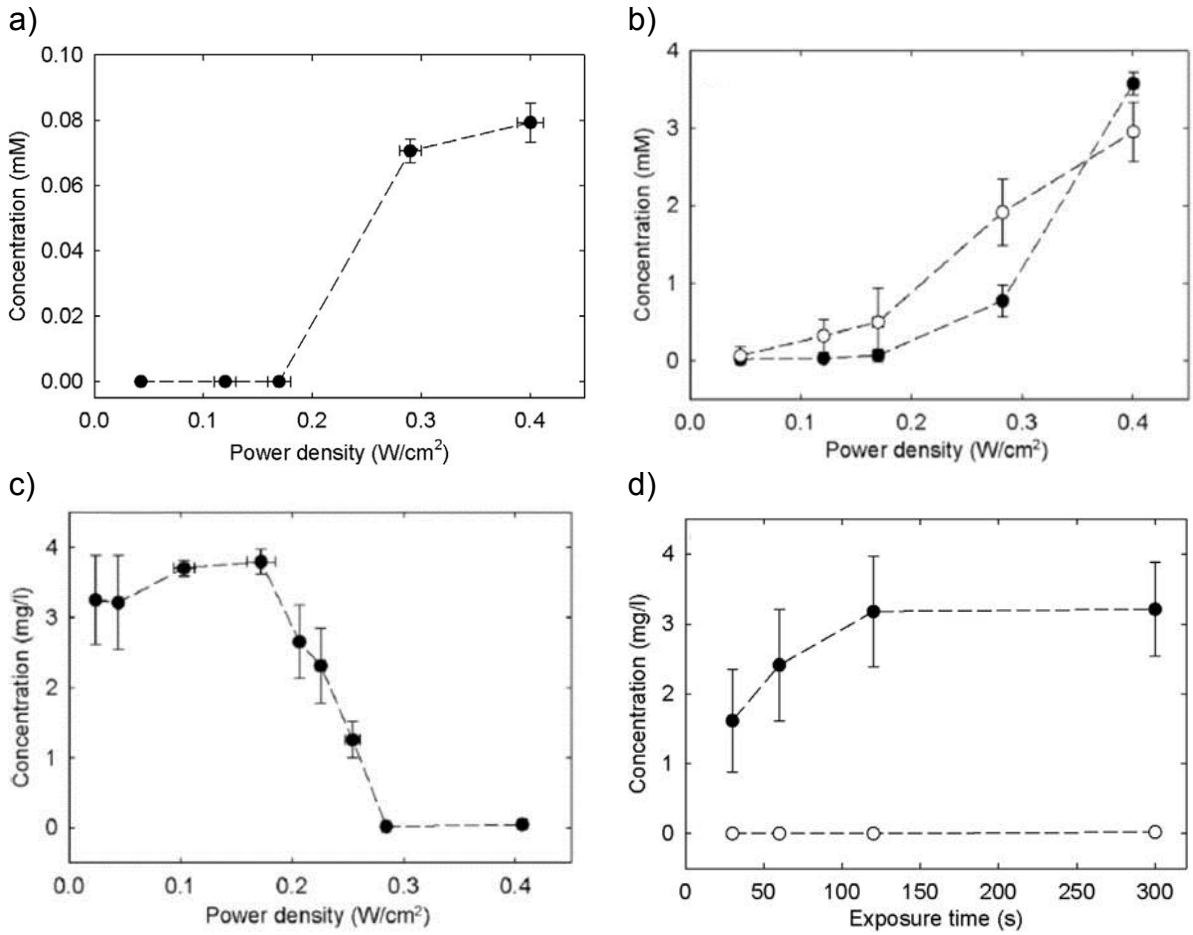


Figure 9: Aqueous chemistry in plasma-treated PBS varying discharge power density:
(a) hydrogen peroxide and (b) nitrite (black) and nitrate (white)
Ozone chemistry in plasma-treated PBS:
(c) varying discharge power density at a fixed exposure time of 300 s and
(d) varying exposure time at fixed power densities of 0.05 W/cm^2 (black) and 0.30 W/cm^2 (white)

The reactions above show us that reactive species not only dissolve in the liquid but also react with the liquid and the newly generated species (as seen above) themselves interact with the cells. Once the reactive oxygen or nitrogen species (RONS) reach the cell, they might activate cell surface receptors and propagate the opening of pores and membrane channels. In case RONS gain access to different cell compartments, the levels of RONS attained in the cell are crucial for their influence on cell fate. At physiological low levels, RONS function as 'redox

messengers' in cellular signalling and regulation, whereas excess ROS induce varying oxidative damage to proteins, lipids and DNA.

In turn these alterations:

- inactivate metabolic enzymes, ionic pumps and structural proteins
- disrupt cell membranes and break nucleic acids,

resulting in the dysfunction of multiple cellular processes. [53, 54]

The final concentration of solved ozone in, for example, swimming pools must not exceed 0.05 mg/L to prevent any kinds of skin irritation. In our experimental setup the portion of solved ozone in the liquid around the sample is therefore negligible. In cell culture models 3 % hydrogen peroxide would be lethal for both bacteria and fibroblasts. [55] A 3% solution of hydrogen peroxide is widely used as a disinfection agent in domestic areas or for contact lenses, as a bleaching agent in dentistry and also for hair. Nitric oxide (NO) is an effective inhibitor of lipid peroxidation serving membrane function and integrity. [56] Moreover, several lines of evidence indicate that in human skin NO is involved in the control of wound-healing processes, allergic skin manifestations, microbicidal activity, antigen presentation, proliferation and differentiation of epidermal cells, and in the regulation of innate immune reactions as well as in inflammatory responses. [57-62]

Nitric acid is a corrosive and a powerful oxidative agent in contact with skin. Systemic effects are unlikely, however, and the substance is not considered a carcinogen or mutagen. The lethal dose for humans is 430 mg/kg (oral) and inhalation should not exceed 2.6 mg/m^3 for 15 minutes [datasheet HNO_3 Merck]. Nitrite is present in blood as a stable oxidation product of NO synthesis or resulting

from an intake of nitrite-containing food; as a constituent of sweat, nitrite is found at concentrations up to 15 µM and is assumed to be formed on the skin surface by commensal bacteria. Moreover, nitrite is used medically as broncho- and vasodilating agent and as an antidote against cyanide poisoning. [62-65]

3.5 Plasma devices used in this study

FlatPlaSter 2.0

The SMD technology is the fundamental principle of all devices that were used in this study. The FlatPlaSter 2.0 incorporates the SMD electrode (size 9 x 13 cm^2) into a box made out of Teflon and Polyoxymethylene as shown in Figure 10:

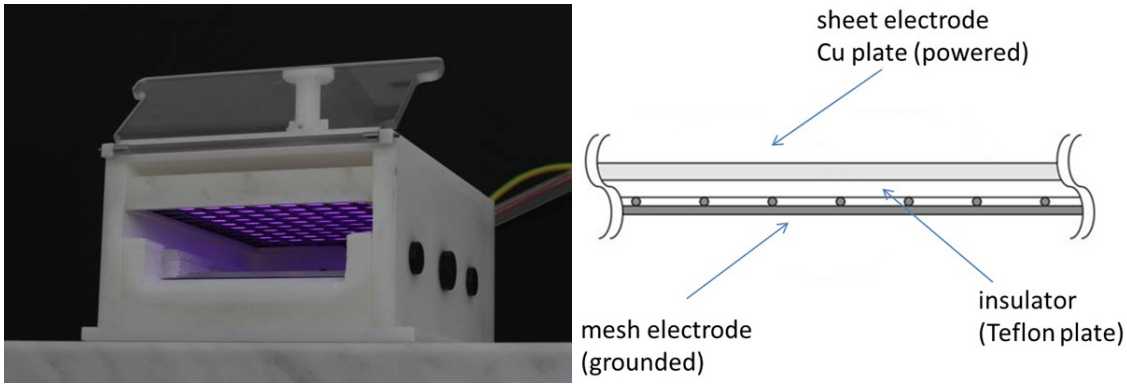


Figure 10: CAP device (FlatPlaSter 2.0), based on the SMD technology. The samples to treat were placed directly beneath the electrode. The position of the electrode is adjustable and was kept at 1 mm above the plate. The door of the device was closed during the CAP treatment, so that the species produced by the plasma were confined inside. [66]

The SMD electrode itself consists of a 0.5 mm thick Teflon plate which is sandwiched by a brass planar plate and a stainless steel mesh grid (line width 2 mm, opening 10 mm, height 1.5 mm). High sinusoidal voltage of 9 kVpp with 1 kHz in frequency is applied between the brass electrode and the mesh electrode. The plasma is produced in many nano- and microdischarges, aided by the strong electric field produced around the wires of the mesh electrode by the potential difference with respect to the other (sheet) electrode. The power consumption for the plasma discharge is 0.02 W/cm^2 and was measured with the Lissajous method using a 1 µF capacitance. [67] Figure 11 shows the ozone evolution of this device.

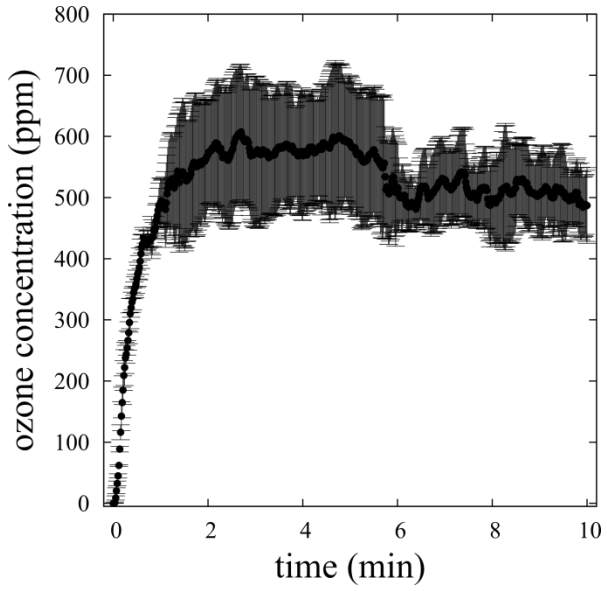


Figure 11: Ozone evolution of the plasma produced by the FlatPlaSter 2.0. The concentration increases monotonically and evens out at a level of approximately 550 ppm.

For the CAP treatment itself with the FlatPlaSter 2.0, the treatment sample is placed inside the box beneath the SMD electrode. A door - installed on one side of the box - further ensures that the produced plasma species do not escape and are confined inside. Transportation of these plasma species to the treatment sample is realized via diffusion - i.e. no additional gas flow is introduced. The distance between the SMD electrode and the treatment sample is variable and can be adjusted. The FlatPlaSter 2.0 was used for the treatment of bacteria in solution [66] and for treatment of primary human dermal fibroblasts.

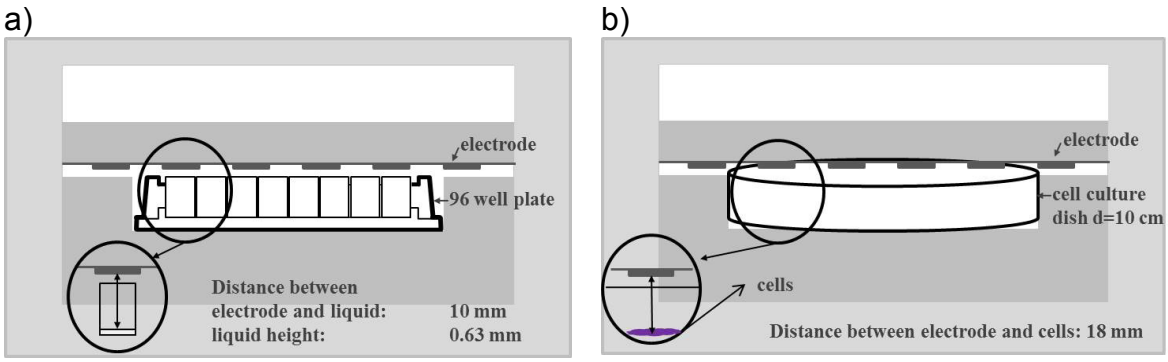
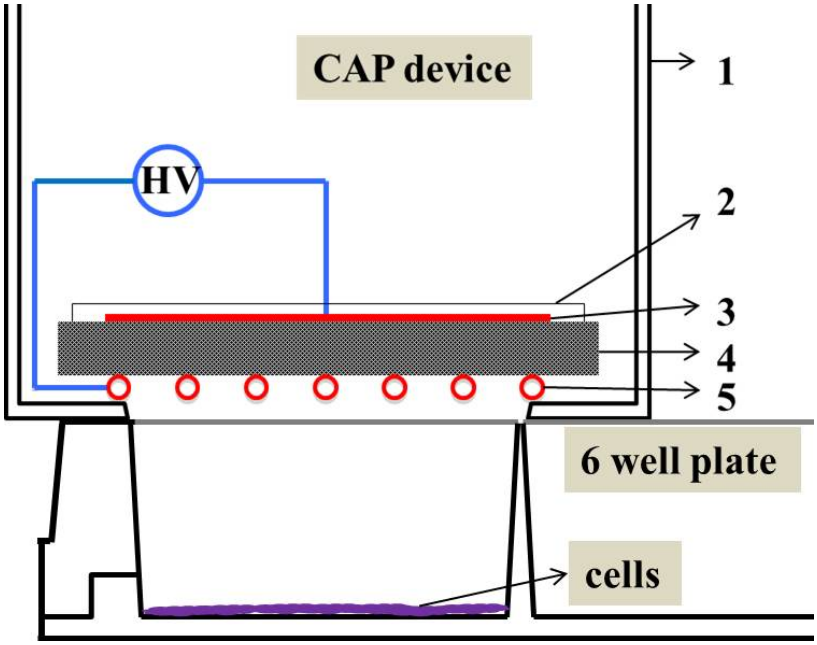


Figure 12: Schematic drawing of the front side of the FlatPlaSter 2.0. Experimental setup for a) bacteria treatment in solution [66] and b) treatment of primary human dermal fibroblasts.

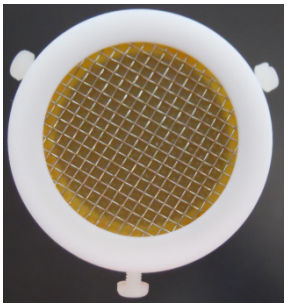
MiniFlatPlaSter

Besides the FlatPlaSter 2.0, different handheld devices were developed and examined (Figure 13). The handheld battery-operated plasma device incorporates an electrode possessing the Surface Micro - Discharge (SMD) Technology [20] and uses the surrounding air for plasma production. The device itself is designed for portable usage and therefore incorporates the high voltage power supply for plasma generation inside. The HV power supply provides a pulsed waveform voltage signal between -2 kV and +5 kV and a repetition rate of approximately 6.75 kHz. [68] The roundly shaped electrode with a diameter of 2.8 cm - located at the top of the device - consists of a glass Epoxy board, which is sandwiched by a copper foil layer and a stainless steel mesh grid. By applying the high voltage signal between the copper plate and the stainless steel mesh, many micro-discharges - and therefore plasma - are produced on the stainless steel mesh. [69] Figure 14 shows the ozone evolution of the plasma produced by the MiniFlatPlaSter. The ozone concentration first increases, but after 10 seconds decreases again immediately.

a)



b)



c)

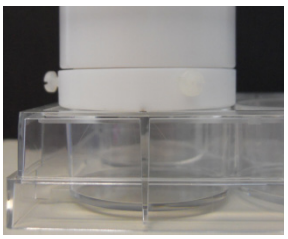


Figure 13:
a) Schematic picture of the CAP device that operates according to Surface Micro - Discharge (SMD) technology and uses the surrounding air for plasma production. It consists of the following five parts: (1): cylindrical tube for housing the plasma generation units, (2): Kapton insulation layer which covers the copper foil layer (3) laminated on the backside of the Epoxy dielectric board (4), (5): mesh electrode made out of a stainless steel grid. By applying high voltage (HV) between the copper plate (3) and the mesh grid (5) the plasma is produced by micro discharges on the side of the mesh grid (5).
b) Image of the electrode.
c) Experimental setup with a 6 well plate. [69]

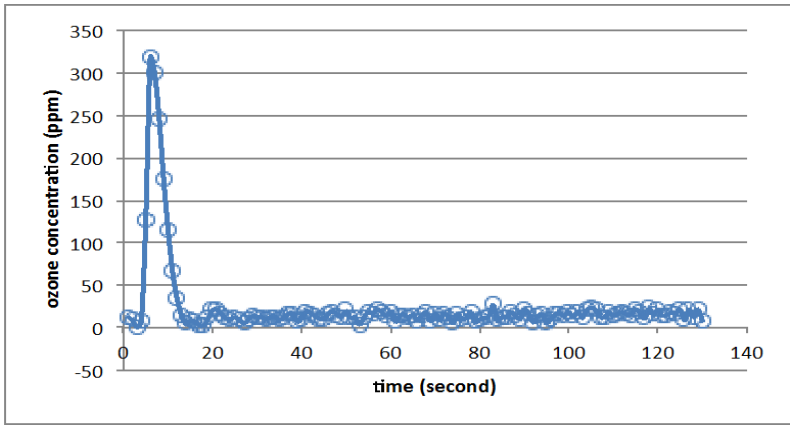
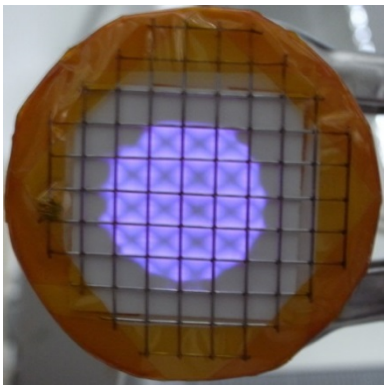


Figure 14: Ozone evolution of the plasma produced by the MiniFlatPlaSter. The ozone concentration first increases, but decreases after 10 seconds again immediately.

Mode transition electrode

The electrode consists of a 0.5 mm thick Al_2O_3 plate sandwiched by a copper high voltage electrode and a wire mesh (separation 5 mm) which is electrically grounded.

a)



b)

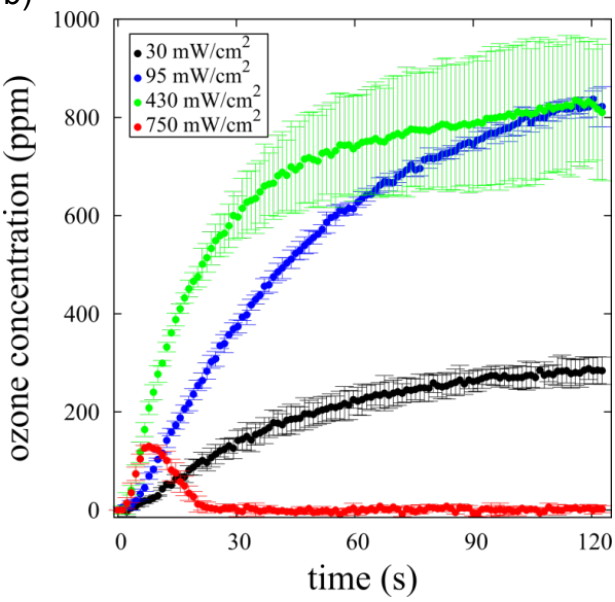


Figure 15:
a) Image of the discharge of the mode transition electrode.
b) Evolutions of ozone concentrations using the mode transition electrode.

The applied voltage was 9.5 kV_{pp} with sinusoidal waveform using a function generator and a high voltage amplifier (Trek Inc. model 10/10B). In order to change the plasma products (chemistry) [35], the frequency of the applied voltage was changed from 60 Hz to 2 kHz. The plasma was produced on the mesh electrode (Figure 15:). From the SMD plasma, the UV power onto the objects is negligibly small in general. Using this electrode, the maximum UV power was 50-80 nW/cm^2 measured by HAMAMATSU UV power meter. This UV power is not high enough to give a bactericidal property using our treatment time on bacteria. Figure 15 shows typical evolutions of ozone concentrations using the mode transition electrode.

3.6 Nosocomial infections and antibiotic resistance

Staphylococcus aureus is a Gram-positive bacterium which colonizes the skin and is present in the anterior nares in about 25–30% of healthy people. [70] Among *Staphylococcus aureus* isolates, Methicillin resistant *Staphylococcus aureus* (MRSA) are one of the most important causative agents in healthcare-associated infections all over Europe. [71] *S. aureus* is the primary cause of lower respiratory tract infections and surgical site infections and the second leading cause of nosocomial bacteraemia, pneumonia, and cardiovascular infections. [72, 73] Infections with *S. aureus* are especially difficult to treat because of evolved resistance to antimicrobial drugs. Resistance to Penicillin and newer narrow-spectrum β-lactamase–resistant Penicillin antimicrobial drugs (e.g. Methicillin, Oxacillin) appeared soon after they were introduced into clinical practice in the 1940s and 1960s, respectively. [74] Penicillin resistance was initially confined to a small number of hospitalized patients, but resistance spread as the use of penicillin increased, first to other hospitals and then into the communities. [75] The World Health Organization (WHO) considered the resistance of bacteria against antibiotics as one of the greatest threats to human health in 2009. In 2014 they released a first global report on antibiotic resistance. The report reveals high levels of resistance to third generation Cephalosporins in *K. pneumoniae* throughout the European Region. In some settings, as many as 60% of *S. aureus* infections are reported to be Methicillin resistant (MRSA). A high percentage of hospital-acquired infections are caused by MRSA or multidrug resistant Gram-negative bacteria. The high proportions of antibiotic resistance in bacteria that cause common infections (e.g. urinary tract

infections, pneumonia, bloodstream infections) in all regions of the world should be an alarming signal to man. The report finds that although most countries in the EU have well-established national and international systems for tracking antibiotic resistance, countries in other parts of the Region urgently need to strengthen or establish such systems. [76]

In 2007, 10 out of 28 countries, mainly southern European countries, the UK and Ireland (high endemic countries), reported MRSA proportions of 25 % or higher. In the northern part of Europe, in particular the low endemic countries (Norway, Sweden, Finland, Denmark, Iceland and the Netherlands) the proportion of isolates resistant to methicillin remained below 2%. [10] In addition to that, the ESGNI-005 Study on intravascular catheter-related infections reported that *S. aureus* was isolated more frequently from samples of hospitals in non-EU countries. 40% of all isolates were resistant to Oxacillin, as were 63.4% of coagulase-negative *Staphylococcus spp.* isolates. [77] Data from the National Nosocomial Infections Surveillance System (USA) suggest that in intensive care units the proportion of *S. aureus* isolates that are resistant to methicillin has increased to 59.5%–64.4%. [78, 79] Recent reports also suggest that community-associated MRSA infections have become the dominant cause of community-associated *S. aureus* skin and soft tissue infections [80, 81]. This fatal trend of increasing resistances faces another inexorable trend - the lack of new antibiotic drugs. [82, 83]

And as if this is not bad enough, there are many bacteria related diseases that are not curable by antibiotics at all. One alarming example here was the 2011 Hemolytic-uremic syndrome (HUS) epidemic in Germany. Enterohemorrhagic *E. coli* (EHEC)

are Gram-negative and therefore possess innately resistance against a lot of antibiotics. Combatting this pathogen with antibiotics has no clear clinical benefit as the secretion of the bacterium is thus prolonged or the course of disease is deteriorated by the increased toxin level.

Already in 1945 Alexander Fleming warned when he obtained the Nobel Prize in medicine for the discovery of Penicillin that bacteria can become resistant against antibiotics. Decades of uncontrolled usage and a flood of antibiotic prescription - especially in non-bacteria related diseases - led to a continuous increase in the emergence of highly resistant bacteria. [84-87] Other reasons for the development of resistances are: the prescription of the wrong type and insufficient doses of the antibiotic for the underlying bacterial infection, the use of a suboptimal antibiotic concentration or an inappropriate short or too long duration of antibiotics. [86]

The “golden ages” of antibiotics are over now and only few pharmaceutical companies are engaged in the development of new antibiotic substances. The reasons are again obvious – it is not a good business model: the approval for drugs for medical use is risky and expensive, the development of resistance to the upcoming drug is inevitable, the time frame of successful administration in patients is therefore getting shorter and shorter and side effects are unavoidable.

3.7 Disinfection and prevention

In 2011 the World Health Day was dedicated to "Combating Antibiotic Resistance" due to the aforementioned reasons. It is clear, that we need new approaches to target these problems so that we do not end up in an era of incurable bacterial

diseases. [88] The first step in this fight is to avoid the spread of pathogens so that nosocomial infections and diseases related to that do not occur in the first place. The most effective method of containment is disinfection of instruments and especially hospital staff and visitors. In hospitals, hand washing practices, which have been shown to be the leading intervention for limiting the spread of nosocomial infections, should be improved to meet recommended guidelines [89] as well as disinfection of instruments and surfaces. There are about 21 million surgical interventions annually, worldwide. The surgeons' disinfection procedure— hand rubbing (3 min) or hand scrubbing (5 min)—has to be repeated many times a day, with a number of negative side effects arising from the mechanical irritation, chemical and, possibly, allergic stress for the skin ([90] and Table 4), not to mention the time required. [20] Table 4 gives a short summary where disinfection is needed and what the standard procedure and agents are at the moment. What they all have in common is that a whole bundle of disadvantages and problems remain.

Table 4: List of disinfection agents and procedures according to the approval list of the Robert Koch-Institute[91]

Purpose	Agent/Procedure	Advantages	Disadvantages
Hands/ skin wound surface	• alcohols • isopropyl alcohol • halogens • povidone-iodine • octenidine • polihexanide • chlorhexidine • silver sulfadiazine • potassium permanganate with additives	• cheap • established	• absorption time • irritation of skin, eyes and respiratory tract • allergies • skin diseases • removal of skin grease • waste
surgical instruments	• ultrasound bathes • liquid agents like aldehydes, phenol derivatives, quaternary ammonium derivatives and oxidative agents, e.g. H_2O_2 • decontamination facilities (thermal)	• cheap • established	• irritation of skin, eyes and respiratory tract • allergies • skin diseases • danger of fire and explosion • risk of infections • risk of burning • not for thermo labile materials and instruments that are difficult to access • waste
surfaces	• aldehydes • phenol derivatives • quaternary ammonium derivatives	• cheap • established	• irritation of skin, eyes and respiratory tract • allergies • skin diseases • waste
room/air	vaporization of • formaldehyde • H_2O_2	• cheap • established	• irritation of skin, eyes and respiratory tract • allergies • skin diseases • danger of fire and explosion • waste

3.8 Objective

The effects of CAP on prokaryotes and eukaryotes were under intensive investigation for the last 20 years. Intensive testing all over the world showed that bacteria of all kinds – including MRSA and EHEC-viruses, fungi and spores can be efficiently inactivated by CAP within a short timeframe, [6, 7, 27, 66, 92-96] thereby opening up a new research field called “plasma medicine”.

Sterilizing temperature sensitive surfaces and medical equipment was the first planned application field of CAP. Due to a close cooperation with dermatologists the treatment of infected and/or chronic wounds and human skin seemed to be a possible area where CAP could be applied.

There are critical numbers of bacteria above which tissue responds with infection. This balance of 10^5 or fewer bacteria/g tissue is also required for wound healing to proceed normally. [97] High levels of bacteria not only result in infection but also inhibit normal wound healing. [98] As plasma has proven its bactericidal efficacy patients should benefit from a CAP treatment by reduction of the bacterial load in open wounds. Chronic wounds in older patients are a big threat and represent a large cohort of patients. Resistant bacteria and decreased healing ability are the main obstacles to overcome here. But before clinical trials could be carried out, experiments had to be done *in vitro*. In this work parts of necessary *in vitro* studies were carried out.

The first part deals with the inactivation of bacteria in solution and the impact of the different components to the bactericidal effect of CAP. Afterwards, the HPRT assay was performed in order to exclude a possible mutagenic potential of CAP.

The last part of the present investigation deals with the effect on skin cells in a wound. Here a primary culture of human dermal fibroblasts was established in order to simulate conditions as realistic as possible.

Finally it was possible to find a “therapeutic window”: the optimal time, which is required to inactivate bacteria and where normal human dermal cells are not yet harmed.

4. Materials and Methods

4.1 Technical devices

Device	Model	Manufacturer
Centrifuges	5471R 4K15	Eppendorf AG, Hamburg, Germany Sigma, Deisenhofen, Germany
CO_2 incubator **Incubator (microbiology)**	HERAcell®	Thermo Fisher Scientific Inc, Waltham, USA
Flow cytometer	FACScalibur ™	Becton Dickinson GmbH, Heidelberg, Germany
Magnetic stirrer	RCT basic	IKA®-Werke GmbH & Co KG, Staufen, Germany
Microplate reader	Asys Expert plus Infinite F200 PRO	Biochrom AG, Berlin, Germany Tecan Group Ltd., Männedorf, Switzerland
Microscopes	Axiovert 25 AxioImager Z.1 Eclipse TS100	Carl Zeiss AG, Jena, Germany Carl Zeiss AG, Jena, Germany Nikon, Düsseldorf, Germany
pH-meter	EL-30	Mettler-Toledo GmbH, Giessen, Germany
Spectrophotometer		
Water bath		

4.2 Chemicals, reagents and cytostatics

Substances	Abbreviation	Manufacturer
96% Acetic Acid		Carl Roth GmbH &Co KG, Karlsruhe, Germany
4,5-Diaminofluorescein	DAF-2	Enzo Life Sciences GmbH Lörrach, Germany
BactoPepton		Invitrogen, Karlsruhe, Germany
Diamidino-phenylindole	DAPI	Roche Diagnostics, Mannheim, Germany
Ethyl methanesulfonate	EMS	Sigma Aldrich, Munich, Germany
Ethanol		Carl Roth GmbH &Co KG, Karlsruhe, Germany

Formaldehyde		Carl Roth GmbH &Co KG, Karlsruhe, Germany
Glycerol		Carl Roth GmbH &Co KG, Karlsruhe, Germany
Hydrochloric acid	HCl	Carl Roth GmbH &Co KG, Karlsruhe, Germany
Hydrogen peroxide	H_2O_2	Carl Roth GmbH &Co KG, Karlsruhe, Germany
Isopropanol		Carl Roth GmbH &Co KG, Karlsruhe, Germany
Methanol		Carl Roth GmbH &Co KG, Karlsruhe, Germany
Phosphate buffered saline	PBS	PAA, Pasching, Austria
Propidium iodide		Sigma Aldrich, Munich, Germany
Select agar		Invitrogen, Karlsruhe, Germany
Spermine NONOate		Sigma Aldrich, Munich Germany
Select Yeast Extract		Invitrogen, Karlsruhe, Germany
Sodium chloride	NaCl	Carl Roth GmbH &Co KG, Karlsruhe, Germany
Sodium hydroxide	NaOH	Carl Roth GmbH &Co KG, Karlsruhe, Germany
Tris		Carl Roth GmbH &Co KG, Karlsruhe, Germany

4.3 Software

Software	Producer
Adobe® Acrobat® X Suite	Adobe Systems incorporated, San Jose, USA
Axiovision Rel. 4.8	Carl Zeiss Microscopy, LLC, Thornwood, USA
EndNote X7	Thomson ISI Research Soft, NY, USA
FlowJo analysis software	Tree Star Inc., Ashland, USA
GraphPad Prism®	GraphPad Software, LaJolla, USA
NIS Elements F 3.2	Nikon Instruments Inc., Melville USA

4.4 Consumables and additives

Substance / material	Abbreviation	Manufacturer
Antibiotic/Antimycotic	A/A	Sigma Aldrich, Munich, Germany
Cell culture inserts for migration assays		ibidi, Munich, Germany
Collagenase Type II		life technologies, Darmstadt, Germany
Dissection instruments		Carl Roth GmbH &Co. KG, Karlsruhe, Germany
Dispase II		life technologies, Darmstadt, Germany
Dulbecco's Modified Eagle Medium GlutaMAX-I, high Glucose	DMEM	life technologies, Darmstadt, Germany
Fetal bovine serum	FCS	life technologies, Darmstadt, Germany
McCoys 5A medium		life technologies, Darmstadt, Germany
Mitomycin C from *S. caespitosus*		Sigma Aldrich, Munich, Germany
Neubauer hemocytometer		VWR International GmbH, Darmstadt, Germany
Penicillin/Streptomycin	P/S	life technologies, Darmstadt, Germany
Petri dishes for bacterial culture		SARSTEDT AG &Co., Nümbrecht, Germany
Phosphate buffered saline	PBS	PAA, Pasching, Austria
RNase A		Sigma Aldrich, Munich, Germany
Serological pipettes, centrifuge tubes		SARSTEDT AG &Co., Nümbrecht, Germany
6-Thioguanine	6-TG	Sigma Aldrich, Munich, Germany
Trypsin		life technologies, Darmstadt, Germany
Tissue culture dishes/flasks/test plates		TPP Techno Plastic Products AG, Trasadingen, Switzerland

Cell culture media and solutions

Media	Components
Cell freezing medium	DMEM + 20 % FCS + 5 %DMSO
Medium for V79	DMEM + 10% FCS + 1% P/S
Medium for primary culture	McCoy's 5 A + 10 % FCS + 1% A/A
Tissue disaggregation solution	Dispase + 1% A/A
Tris buffered saline	2.42 g Tris, 8 g NaCl, pH 7.6 with acetic acid, ad 1000 ml H_2O

4.5 Bacterial culture

ATCC 9637' *Escherichia coli* (kindly provided by Dr. Hans-Ulrich Schmidt, Department Med. Microbiology and Hospital hygiene, Hospital Munich Schwabing) were maintained according to standard protocols. [99, 100] A wire loop was flamed and cooled on a spare sterile agar plate. Using the wire loop, an inoculum of bacteria was streaked across one corner of a fresh agar plate. The wire loop was flamed and cooled again. It was passed through the first streak and then streaked again across a fresh corner of the plate and repeated again to form a pattern. The plate was incubated upside down at 37°C for 16 h until colonies developed. Liquid Lysogeny Broth Medium (LB Medium) was inoculated with a healthy colony, picked from a freshly streaked plate and incubated for 16 hours in the shaking incubator at 37°C and 200 rpm. Cultures were stored for 14 days in the refrigerator at 4°C.

4.6 Cell cultivation

Cultivation and cryopreservation of V79 cells

The standard cell line for the hypoxanthine-guanine phosphoribosyl transferase (HPRT) assay is V79 Chinese hamster lung fibroblasts. They were maintained in DMEM supplemented with 10 % FCS and 1 % P/S under standard cell culture conditions at 37°C and 5 % CO_2. Cells were passaged twice a week. Therefore, cells were washed with PBS, trypsinized for cell detachment and then seeded in fresh medium.

For cryopreservation, cells were harvested at 70 % confluence and dissolved in freezing medium. After gradual freezing for 2 days at -80°C, cells were stored at -180°C in liquid nitrogen.

Primary cell culture

Primary human dermal fibroblasts (PHDF) were isolated from adult donors which underwent resection of melanoma.

Excised specimen were transported in McCoy's 5a medium supplemented with 1% A/A, transferred into tissue disaggregation solution and incubated at 4°C over night. The dermis was then peeled off the epidermis and cut into small pieces (>0.5 cm). Tissue pieces were then digested in Trypsin supplemented with Collagenase (2 mg/ml) for 1 hour at 37 °C. Cells were strained by pipetting and centrifuged afterwards at 400 x g for 10 minutes. The supernatant was discarded and the cell pellet was obtained in primary culture medium. Cells were seeded and cultured under

standard cell culture conditions at 37°C and 5 % CO_2. Cells were used until passage 12 and then discarded.

In addition to that normal human dermal fibroblasts (NHDF) were purchased from Promocell (Heidelberg, Germany) and cultured in McCoy's (+ 10 % FCS + 1 % A/A) under standard cell culture conditions at 37°C and 5 % CO_2. NHDF cells were passaged and cryopreserved like V79 cells.

4.7 Treatment of bacteria in solution with the FlatPlaSter 2.0

Plasma application and experimental setup

In the setup used in this study, bacteria were treated with the FlatPlaSter 2.0 in 20 µl of Tris buffered saline (TBS, pH 7.6) in wells of a 96 well plate. The 96 well plate was treated with CAP with a distance between the electrode and liquid surface of ~ 10 mm. The liquid height in the well was ~ 0.63 mm (Figure 12 a).

Cellnumber tests

10^2 – 10^8 cells of *E.coli* in 20 µl TBS were brought into wells of a 96 well plate and treated with CAP for 1, 2, 3, 4, 5 and 8 minutes. Afterwards the samples were incubated for 1 hour at room temperature, spread on agar plates (in different dilutions) and subsequently incubated at 37°C for 18 h, so that the surviving bacteria could reproduce and form colonies. Dilutions of untreated bacteria were performed as a control for the initial cell number. The killing efficacy was calculated from the initial cell number and the total count of colony forming units (CFUs).

Detection of reactive oxygen species

Hydrogen peroxide (H_2O_2) emergence in solution during CAP application was detected with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay (Molecular Probes) for several time points. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resorufin. Excitation was detected at 550 nm and emission at 595 nm with a microplate reader (Infinite® 200 Pro, Tecan). The Amplex® Red Hydrogen Peroxide/Peroxidase Assay was performed according to the manufacturer's protocol with CAP treated bacteria in TBS and TBS alone.

Detection of nitrites and nitrates

The Nitrate/Nitrite Colorimetric Assay (Cayman Chemical Company) is based on the reaction of Griess Reagents with nitrates (nitrites are first reduced to nitrates) which then forms a deep purple azo compound. The assay was performed according to the manufacturer's protocol with the supernatants of plasma treated *E.coli* in TBS and TBS alone. The absorbance was measured photometrically at 550 nm with a microplate reader (Infinite® 200 Pro, Tecan).

Reference experiments with H_2O_2 and NO

Reference experiments with H_2O_2 (Carl Roth) and a chemical NO donor (DEA NONOate, Sigma Aldrich) were performed. TBS containing 10^4 *E.coli* cells was incubated for one hour with H_2O_2 and DEA NONOate separately and in combination to final concentrations in the range of the measured concentrations after the CAP

treatment times of 20 µl TBS containing *E.coli*. Samples were then spread on agar and incubated over night at 37°C. CFUs were counted to control the bacterial reduction.

Fluorescence proof of intracellular NO

To detect NO in injured bacteria, 5 x 10^6 bacteria in 20 µl TBS were filled in wells of a 96 well plate and treated with CAP for 3 min. After the treatment bacteria were stained with DAF-2 (Enzo Life Sciences) at a final concentration of 10 µM for 1 hour at 4°C in the dark. Bacteria were fixated on microscope slides and nuclei of bacteria were additionally stained with 10 nM 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes). As controls, living cells and cells treated with a chemical NO donor (DEA NONOate, Sigma Aldrich) were prepared. Samples were visualized on an Axioimager fluorescence microscope (Zeiss) equipped with a Zeiss Axiocam camera and Zeiss objectives and filters. Light was collected through a 63 x 0.4 NA water immersion objective. Fluorescence was excited using a 358 nm band pass filter for DAPI and 465 - 495 nm for Triazolofluorescein. Emission was detected by a 440 - 480 nm band pass filter (DAPI) and a 505 - 550 nm band pass filter (Triazolofluorescein). Images were captured from three randomly selected areas for ten different sets of experiments and data were analysed using Axiovison 4.8 software (Zeiss).

4.8 Treatment of dermal fibroblasts with FlatPlaSter 2.0

Cell viability

For the investigation of cell viability after the treatment with CAP 1 x 10^5 cells were seeded in a 6 well plate and grown over night. Cells were treated with plasma without medium covering them (fresh medium was added immediately afterwards). Controls were kept without medium for the same duration as CAP treatment. Cells were incubated for 48 h at standard cell culture conditions and then washed, trypsinized and counted with a hemocytometer.

Migration

The scratch assay was performed with primary cells and NHDF cells grown to confluence in 6 well plates. To see only migration proliferation was inhibited by incubating cells for 3 hours with 2 g/l Mitomycin. Then cells were washed, treated with plasma (FlatPlaSter 2.0) and at the end a scratch was set with a 200 µl pipette tip. Fresh medium was added cells were then cultivated under normal conditions for 48 hours. Images were made and gap size was measured at three randomized positions after the scratch and after 24 hours and 48 hours of incubation. Control cells were not plasma treated.

Flow cytometry

Cell cycle analysis was carried out by flow cytometry. Cell nuclei containing different amounts of DNA in the cell cycle phases were stained with propidium iodide

(PI). The G1 phase is featured by two complete sets of chromosomes (2N). During S phase, DNA is synthesized and the DNA amount is intermediate between G1 and G2. In G2 phase, doubled DNA content defines a tetraploid stage (4N). Following RNA and protein synthesis, G2 phase culminates in mitosis (M phase). Regarding DNA amount, G2 and M phase are indistinguishable. Apoptotic cells with fragmented DNA appear as subG1 population.

Cells were seeded in 10 cm tissue culture dishes (5×10^5 cells/ dish), allowed to attach overnight, and CAP treated (FlatPlaSter 2.0) for the indicated times. Controls were treated equally. For FACS analysis cells were washed twice in phosphate buffered saline (PBS) and fixed in ice-cold 70 % methanol at 4°C for at least 2 h. Afterwards cells were washed with PBS and then incubated with 100 µg/ ml of RNase A for 20 min at 37°C and stained with PI (50 µg/ ml). Cell cycle distribution was analysed using the BD FACS Calibur counting 10,000 events per determination. Doublet discrimination and analysis of cell cycle distribution was performed with FlowJo analysis software.

4.9 Mutagenicity of CAP (MiniFlatPlaSter)

Reference experiments with *E.coli*

To evaluate a reasonable duration of CAP-treatments for the mutagenicity tests, first of all the reduction rate of 'wet' *E.coli* by the MiniFlatPlaSter was measured. *E. coli* K1 ATCC 10538 were used as a model system. A suspension of *E. coli* in phosphate-buffered saline (PBS) with a density of approximately 2 x 10^8 cells per ml was prepared as a stock bacterial suspension. A volume of 0.3 ml of this stock

suspension was then distributed homogeneously to Müller-Hinton agar plates (culture area approximately 8.5 cm^2) and immediately treated with CAP without drying (agar surface was wet). For comparison, the experimental setup for the bacterial tests was chosen to be as close as possible to the conditions for the experiments with V79 cells:

a. the bacteria on the agar were not dried before the CAP treatment, they were treated immediately after distribution, meaning that they were still 'wet'.

b. the distance between the electrode and the agar surface was set to 17.5 mm.

c. the volume between the CAP device and the agar surface was closed by a plastic ring adapter which had the same inside diameter of a well in a 6-well plate.

d. the *E.coli* density on the agar plate was chosen to be approximately 10^6 colony-forming units (cfu)/cm^2, which means that the target surface on the agar plate was covered with bacteria to approximately 10 %, similar as for the cell experiments.

CAP-treatment times of 120, 180 and 240 sec were used. Each CAP-treatment was repeated three times and the mean of the standard error was then given for the statistical variability. After the CAP-treatment, the agar plates were incubated overnight at 37°C. Colony forming units in the CAP-treated area were then counted, and the reduction due to the CAP-treatment was then calculated.

Hypoxanthine-guanine phosphoribosyl transferase (HPRT) assay with V79 cells

The protocol for the mutagenicity test was modified according to Davies *et al*. [101] In this setup 10^5 V79 cells were seeded in wells of a 6-well plate and cultured in DMEM supplemented with 10 % FCS and 1 % p/s under standard cell culture conditions (37°C and 5 % CO_2) for 24 hours. Before the CAP-treatment, the medium was removed until no visible liquid was seen. The cells were then treated with the MiniFlatPlaSter (see Figure 13) for 30, 60, 2 x 60, 3 x 60 and 4 x 60 seconds or for 30 seconds every 12 hours (in total five times). Positive controls were prepared by treating the cells with 0.3 mg/ml ethylmethane sulfonate (EMS, Sigma Aldrich) in full medium for three hours. This concentration was chosen in order to guarantee an optimum ratio of cell survival and mutant frequency. [102] Negative controls were prepared by removing the medium, but without applying a CAP- or EMS-treatment. After treatment the cells were cultured in complete medium for three days. In order to observe cell behaviour after the CAP-treatment, microscope images of the cells were taken before, immediately after, and 1 hour, 18 hours, 24 hours, 48 hours, and 72 hours after the 240-sec CAP-treatment. After treatment the cells were cultured in complete medium for three days.

At day 4 the cells were washed, trypsinized and counted with a haemocytometer. This cellnumber was also an indicator for cell viability. To assess the plating efficiency (PE), 250 cells per 6-cm dish were re-plated and maintained under standard cell-culture conditions. After another five days colonies were counted. To estimate the mutant frequency (MF), 10^5 cells per 10 cm dish were re-plated and cultured. After five days the medium was supplemented with 5 µg/ml of the selective

agent 6-TG (Sigma Aldrich). Mutant colonies were counted after 8-10 days of incubation.

4.10 Investigation of mode transition

Ozone measurement

Ozone is one of the important products from the SMD plasmas. Here, by an absorption spectroscopy the ozone concentration was measured using UV light at 254 nm in wavelength from a Hg/Ar lamp. Using the mode transition electrode, a quartz glass tube with 30 mm diameter and 20 mm height was placed below the electrode. The glass tube was on a ceramic plate. By the electrode, the glass tube and the ceramic plate, a closed volume was formed. At a height of 10 mm from the ceramic plate, the ozone concentration was measured. Environmental humidity is one parameter which can change the plasma chemistry. This ozone measurement was carried out under 20-23°C with 40-50 W relative humidity. When cell cultures or agar plates were treated by the SMD plasma, an increasing in humidity in the closed volume was expected. To simulate the condition, we measured the ozone evolutions with different powers and different humidities. “Dry” condition means the plasma production using the ambient mentioned above. For “Wet” condition, 50 microliter of distilled water was distributed on the ceramic plate. Unfortunately, the humidity inside the closed volume could not be measured because the volume was too small using our measurement system.

Bactericidal effect

A suspension of *E. coli* (DSM 1116) in phosphate-buffered saline (PBS) with a density of approximately 1 × 10^8 cells per ml was prepared as a stock bacterial suspension. A volume of 100 µl of this stock suspension was then distributed homogeneously to Müller-Hinton agar plates and dried for 30 minutes. For the mode transition electrode the volume between the CAP device and the agar surface was closed by a plastic ring adapter which had almost the same inside diameter and height of a well in a 6-well plate. Bacteria samples were then treated for 15, 30, 45 and 60 seconds with CAP. Colony forming units (CFUs) were counted 24 hours after incubation at standard conditions.

Viability of dermal fibroblasts

For the investigation of cell viability after the treatment with the mode transition electrode experiments were carried out in the same manner as described in 4.8 for the FlatPlaSter. The experimental setup is similar to the setup with the MiniFlatPlaSter (Figure 13 b). The distance between the electrode and the sample surface of interest was 17.5 mm and the treatment volume was closed.

4.11 Statistical analysis

All statistical significances were evaluated using one or two factor analysis of variance (ANOVA) followed by Bonferroni post-test. Differences were considered significant at $P < 0.001$. Experiments were repeated at least three times if not stated otherwise.

5. Results

5.1 Treatment of bacteria in solution with FlatPlaSter 2.0

Studies with the FlatPlaSter 2.0 and other plasma devices already showed that a lot of different pathogens could be inactivated within a short time on agar. As wound treatment is one of the intended application fields of CAP, inactivation experiments were carried out in a small amount of liquid which should simulate the wet wound environment. *E.coli* were treated with the FlatPlaSter 2.0 for indicated times in 20 µl of TBS. The height of the liquid in a well of a 96 well plate was 0.63 mm and the distance to the electrode was 10 mm (also see Figure 12 a).

CellnumberTests

The initial cell density of *E.coli* in the liquid had a strong impact on the inactivation efficacy. A time dependence in strong correlation to the cell density was demonstrated (Figure 16): up to densities of 10^5 cells / 20 µl high reduction rates of up to 5 log steps were achieved in less than 3 minutes of CAP application. At a concentration of 10^6 cells / 20 µl a 6 log reduction was achieved in less than 6 minutes. In contrast, for higher cell densities above 10^7 cells / 20 µl the killing efficiency was not time dependent anymore and almost no reduction was measured for CAP treatment times of up to 8 minutes.

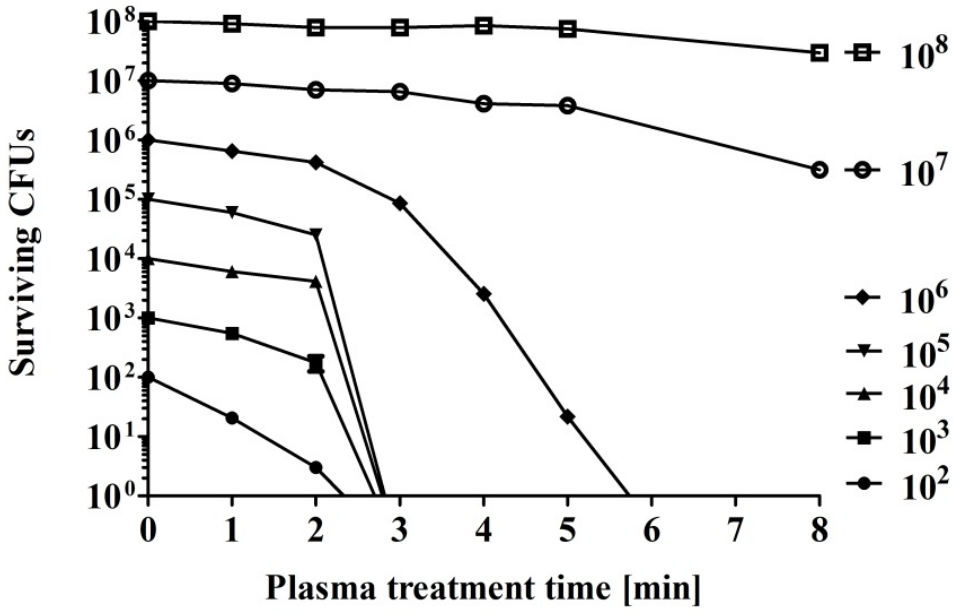


Figure 16: Kinetic inactivation curve of 10^2 – 10^8 *E.coli* cells in 20 µl TBS. The bacteria were treated for 1, 2, 3, 4, 5 and 8 minutes with CAP. A 5 log reduction was achieved for 3 minutes of CAP application for initial cell densities of 10^5 per 20 µl or lower. For cell densities of 10^7 / 20 µl and higher almost no reduction was achieved for CAP treatment times of up to 8 minutes. [66]

Peroxides in liquid

The emergence of peroxides in 20 µl TBS and TBS containing 10^6 *E.coli* after different CAP treatment times was assessed (Figure 17). The results clearly show that the content of hydrogen peroxide in the treated liquids (TBS with/without *E.coli*) increased with longer CAP application times. However, reference experiments with hydrogen peroxide show that the measured hydrogen peroxide concentrations after CAP application are not high enough to inactivate microorganisms (Figure 19 a).

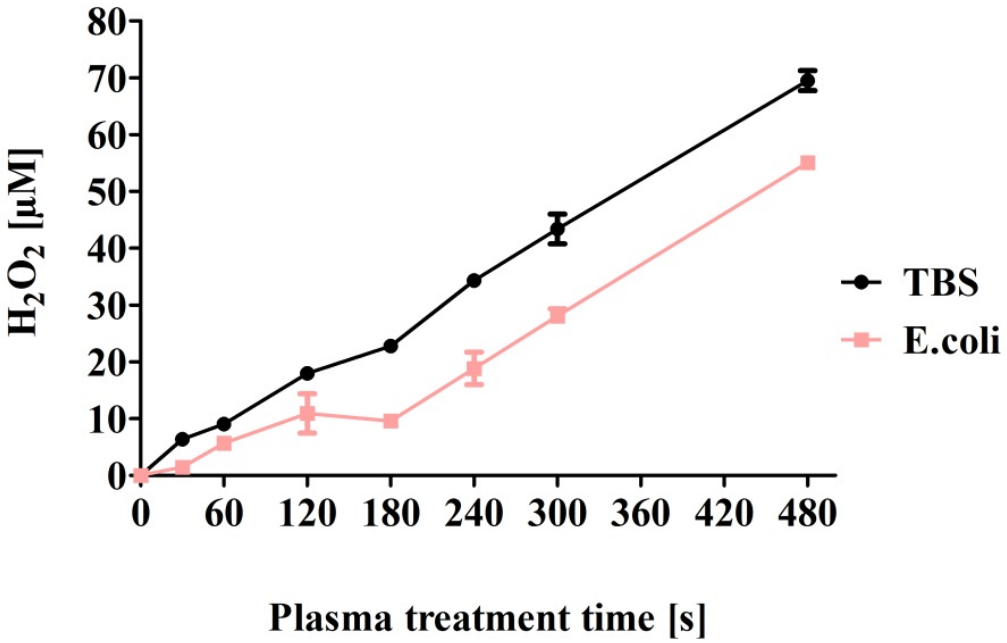


Figure 17: Emergence of peroxides in 20 µl TBS and TBS containing 10^6 *E.coli* after different application times. The peroxide content of TBS and TBS containing *E.coli* increases continuously. In TBS containing *E.coli* the peroxide level is slightly below the level of TBS alone. [66]

Nitrates and nitrites in liquid

The total nitrite and nitrate content in 20 µl TBS and TBS containing 10^6 *E.coli* for different CAP application times was measured (Figure 18). The results show that the nitrite and nitrate content of both treated liquids (TBS with/without bacteria) is significantly increased after CAP application: for a treatment time of 4 min (where a 5 log reduction of *E.coli* was achieved) the concentration reached a value of 950 µM and 1420 µM for a treatment time of 8 min. However, reference experiments with NO possessing concentrations of up to 1500 µM did not reduce bacteria significantly (Figure 19 b and c).

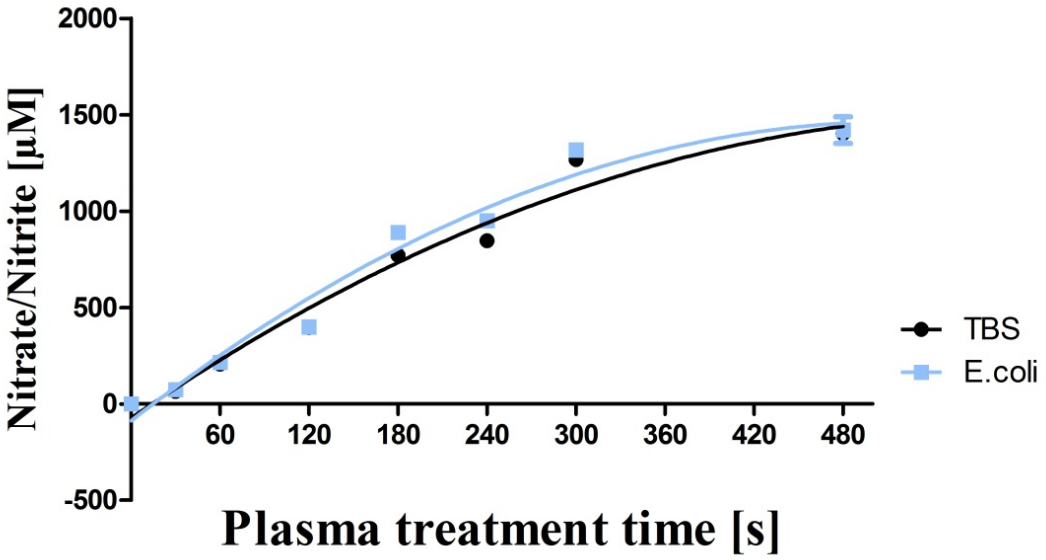


Figure 18: Total nitrite and nitrate content in 20 µl TBS and TBS containing 10^6 *E.coli* after different plasma application times. The Nitrate/Nitrite level is similar for TBS and TBS containing *E.coli*. The curves are a nonlinear fit to the data. Error bars show the standard deviation. It appears that the nitrate/nitrite concentration may become saturated and there is no reduction when the medium contains *E.coli* – this is in contrast to the Hydrogen Peroxide concentration shown in Figure 17. [66]

Combination of H_2O_2 and NO

Reference experiments with a combined application of NO and hydrogen peroxide show a much higher inactivation effect on *E.coli* than a treatment with NO or H_2O_2 alone. The concentrations of nitrates and H_2O_2 that were measured after CAP application had no bactericidal effect in the reference experiments with the chemical NO donor NONOate and H_2O_2 (Figure 19 a). But a reduction of 4 log steps can be achieved with 1 mM of NONOate in combination with 1.5 mM of H_2O_2 (Figure 19 b).

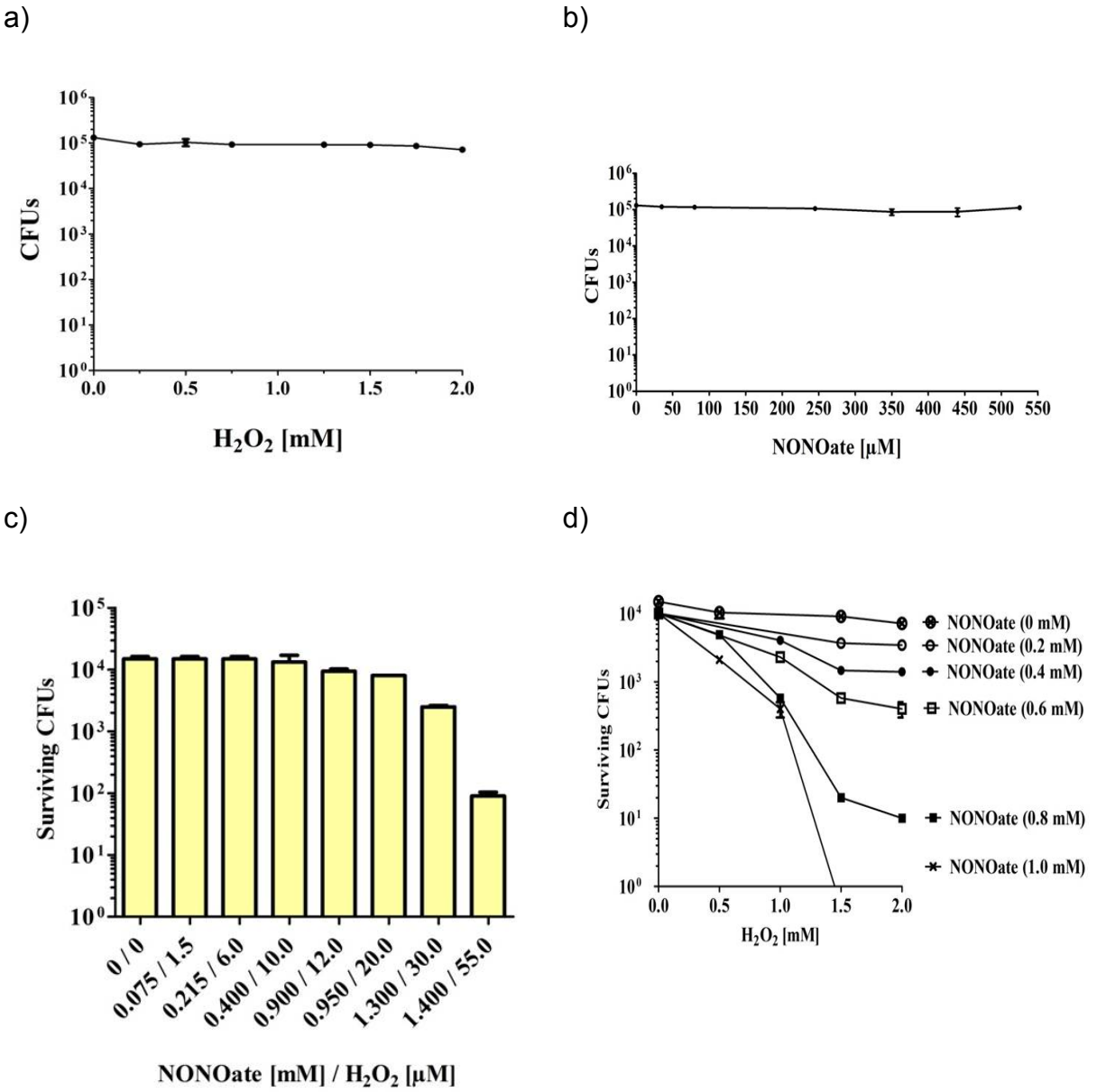


Figure 19: Reference experiments with a) H_2O_2 alone, b) NONOate alone and c) combination of H_2O_2 and NONOate. The concentrations in c) correspond to those obtained after 1, 2, 3, 4, 5, 6 and 8 minutes of plasma application. d) Reference experiments with explicitly higher concentrations of NONOate and H_2O_2. [66]

Direct evidence of intracellular NO in *E.coli*

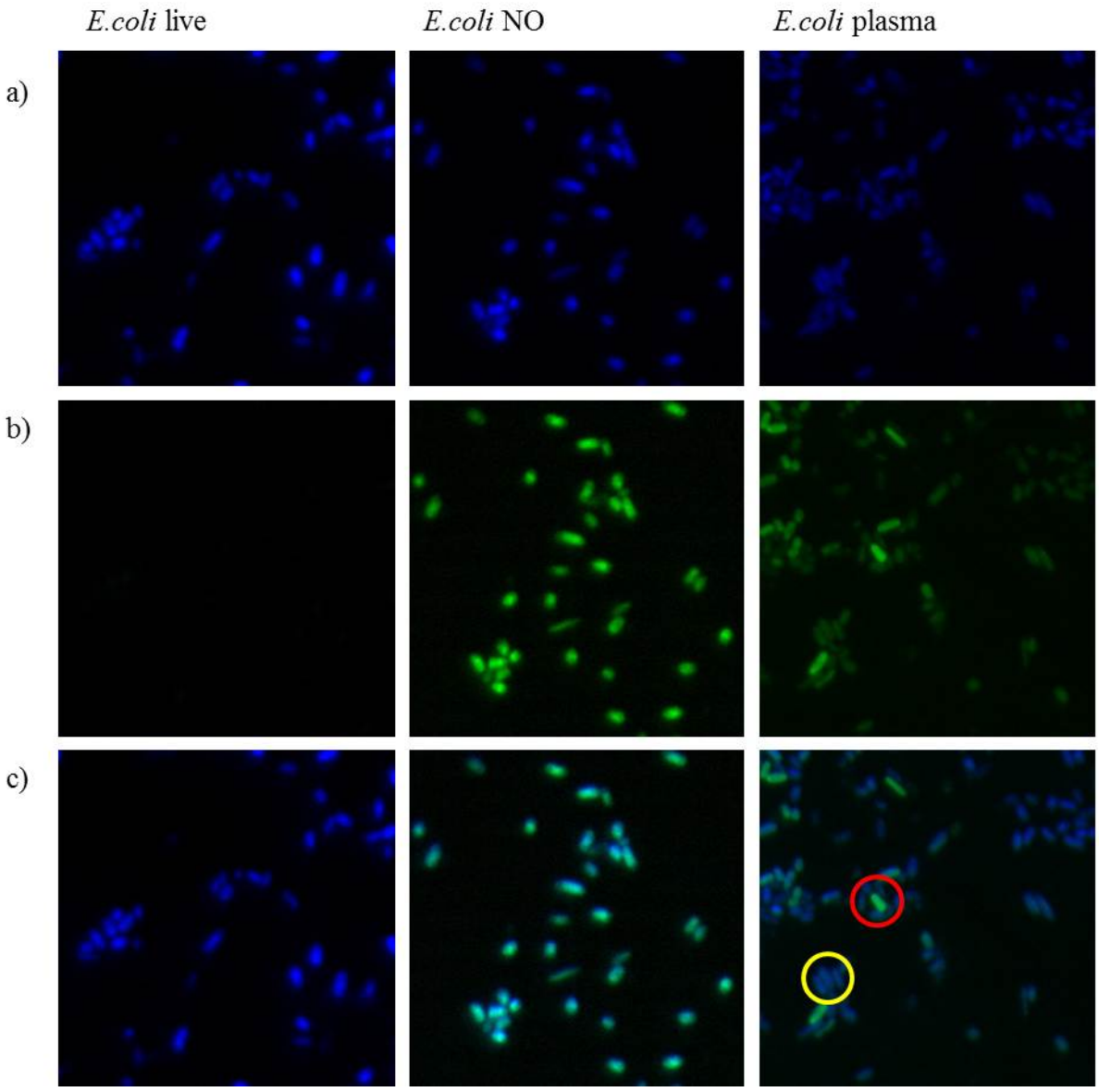


Figure 20: Living *E.coli* cells (*E.coli* live), E.coli cells treated with a chemical NO-donor (*E.coli* NO) and *E.coli* cells, treated with plasma for 3 minutes (*E.coli* plasma)
(a) Channel 1: DAPI staining; (b) Channel 2: DAF-2 staining; (c) Channel 1 + 2 merged.
Nuclei of all bacteria are stained by DAPI (blue), bacteria that took up NO are stained by DAF-2 (green). Plasma treated bacteria fluoresce green like those which were treated with a chemical NO donor and in contrast to the untreated, living *E.coli* cells. This means that the plasma treated bacteria took up NO during or as a consequence of the plasma treatment. The merged image of plasma treated *E.coli* clearly shows that the uptake of NO is not regularly. The red circle shows a bacterium which took up much more NO than those in the yellow circle. [66]

A sensitive fluorescent probe (DAF-2) was used to detect NO in cells in real-time (Figure 20): controls of living cells show that *E.coli* cells are not stained by DAF-2 in the absence of a NO-donor. CAP treated bacteria clearly possess green fluorescence in the same way as bacteria, which were treated with a chemical NO-donor. As DAF-2 is only converted to DAF-2T in the presence of NO and O_2 the green fluorescence of CAP treated bacteria is a direct evidence of NO being absorbed by cells during or after plasma application.

Conclusion

The first step towards wound disinfection is to investigate the different influences of CAP on microorganisms. Here it was shown that the initial cellnumber plays an important role. The density of cells in the liquid limits the inactivation property at a certain point. In combination with the results of the H_2O_2 and Nitrate/Nitrite experiments we assume that there is a time point, where the reactive species, which are responsible for the bactericidal action, do no longer diffuse in sufficient concentrations into the liquid.

5.2 CAP treatment of dermal fibroblasts with FlatPlaSter 2.0

As the aim of this part of the study was to test the safety of CAP experiments were performed with primary cells that were not immortalized. Fibroblasts from fresh skin of 11 adult donors and NHDF cells (Promocell) were cultured and used for examinations. Dermal fibroblasts were treated for indicated times with the

FlatPlaSter 2.0 without medium covering the cells (for experimental setup see Figure 12).

Viability test

Primary human dermal fibroblasts (PHDF) and NHDF cells were treated with plasma and the cellnumber was counted 48 hours afterward. The cellnumber decreases with increasing treatment time. Cellnumbers are only significant (P<0.001) decreased for treatment times of 60 seconds and longer.

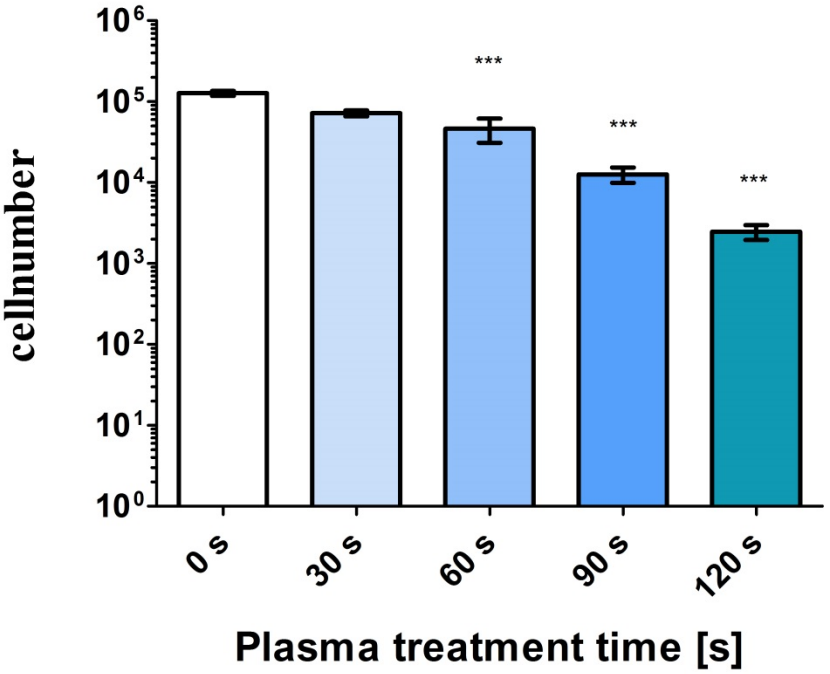


Figure 21: Counted cell numbers 48 hours after different CAP treatment times. Cellnumber was significant (P<0.001) decreased after CAP treatment times of 60 seconds and longer.

Migration

The migration ability after CAP treatment in fibroblasts was investigated. A scratch was originated with a 200 µl pipette tip and proliferation stopped by incubation with mitomycin for 3 hours. Cells were then CAP treated and cultured under standard conditions for 48 hours. Pictures were taken after 24 and 48 hours.

The migration of dermal fibroblasts is slowed down with increasing CAP treatment time. After 24 hours differences in the recovered gap area can be detected. But after 48 hours only cells that were treated for 120 seconds were not able to close the gap. Strikingly, the gap was even more distinct 24 h and 48 h post-treatment for 120 seconds possibly due to detachment of cells.

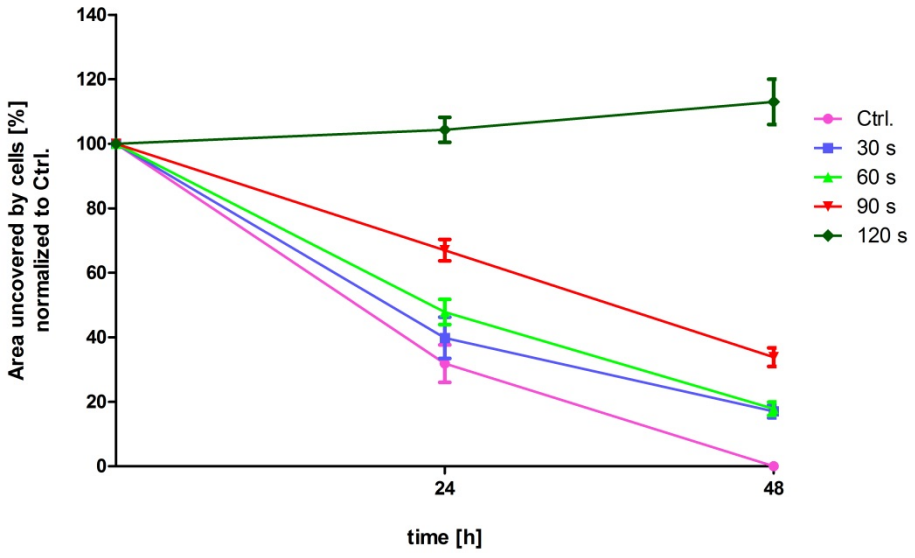


Figure 22: Primary human dermal fibroblasts and NHDF cells were CAP treated and migration of the cells was monitored over 48 h. Gap size was initially measured and after 24 h and 48 h.

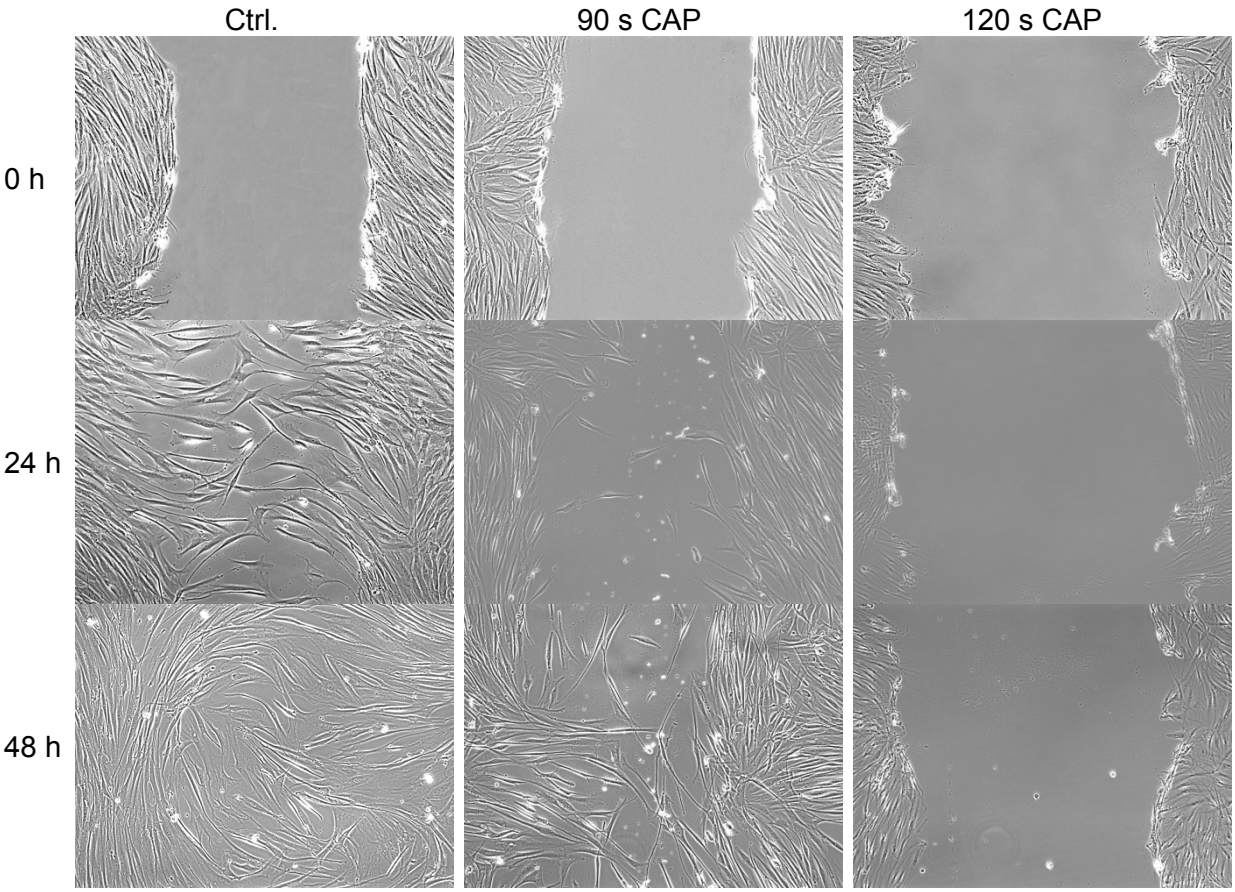


Figure 23: Pictures illustrating the gap directly after the treatment with CAP compared to migration in CAP treated and control cells after 24 h and 48 h.

Cell cycle analysis

Based on these findings, we assumed that CAP has an influence on the regulation of the cell cycle progression. Thus, cell cycle progression of dermal fibroblasts was analysed 4 h, 24 h, 48 h and 72 h after CAP exposure. A treatment time of 90 seconds and higher resulted in a significant arrest in the G2 / M phase of the cell cycle 24 hours after CAP application and persisted for at least 72 hours. Here, up to a factor two higher amounts of cells in the G2 / M phase was found for the CAP treated cells compared to the control cells.

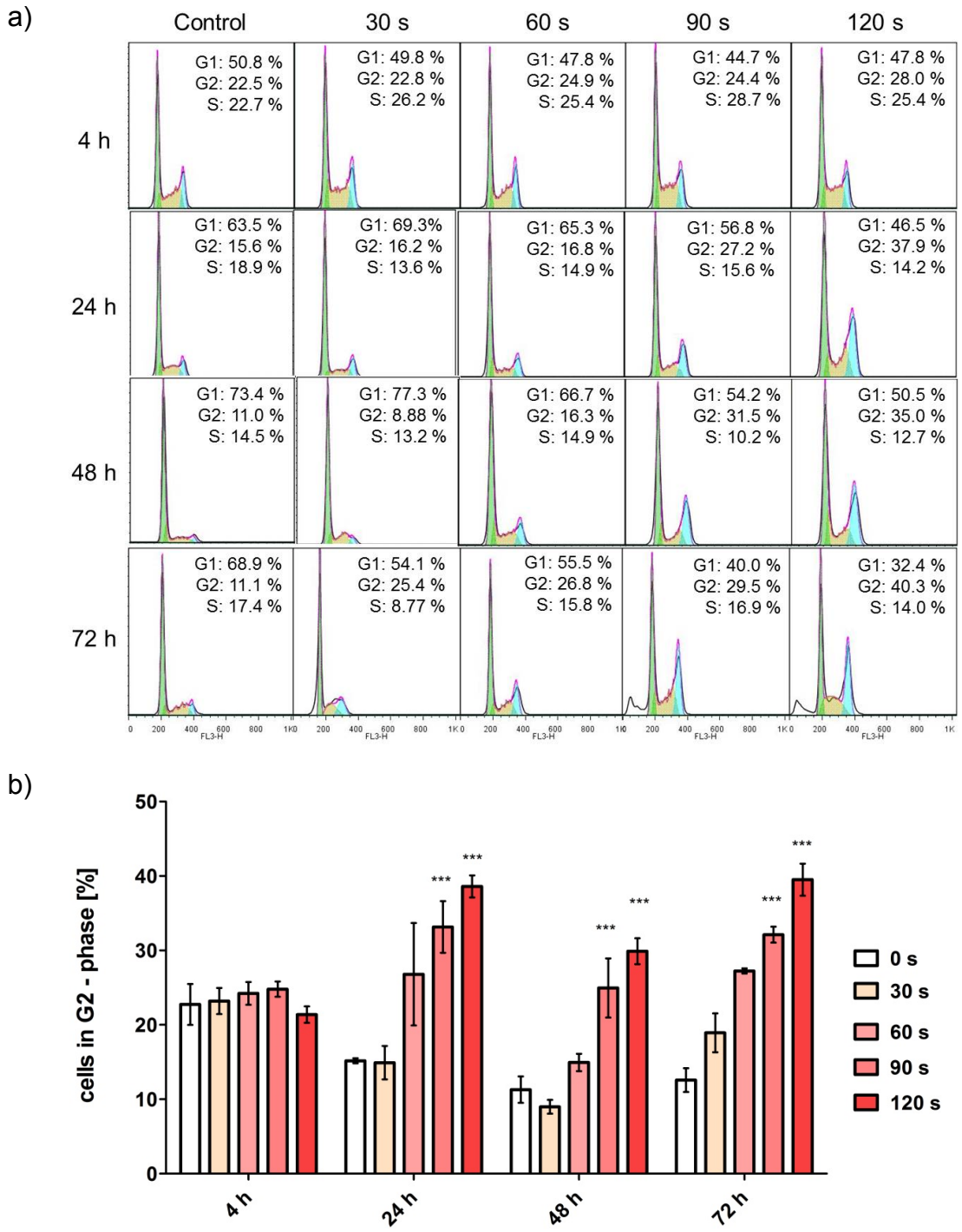


Figure 24: CAP treatment induces G2 / M phase cell cycle arrest in dermal fibroblasts after 90 s.
a) Representative cell cycle distribution of dermal fibroblasts after CAP treatment. Treatment was performed only with a thin film of liquid covering the cells. Cell cycle distribution was analysed using flow cytometry.
b) Cells in G2 / M phase [%].The observed arrest in the G2 / M phase is statistical significant after 24 hours for 90 s CAP treatment and 120 s and lasts up to 72 hours after CAP treatment. P<0.001

Conclusion

After the examination of the bactericidal efficacy of CAP it was necessary to test the safety on human skin cells. Results of proliferation, migration and cell cycle analysis with primary human dermal fibroblasts showed that there is an effect due to higher doses of CAP but that this is not persistent for long time and cells are able to rejuvenate.

5.3 Mode transition electrode

Different power modes result in different chemistry regimes – i.e. the produced reactive species shift from an ozone- to a nitrogen- based chemistry. To assess the impact of these different chemistry modes on as well the bactericidal effect as on the safety towards eukaryotic cells i.e. primary fibroblasts, different power modes were evaluated by using the mode transition electrode.

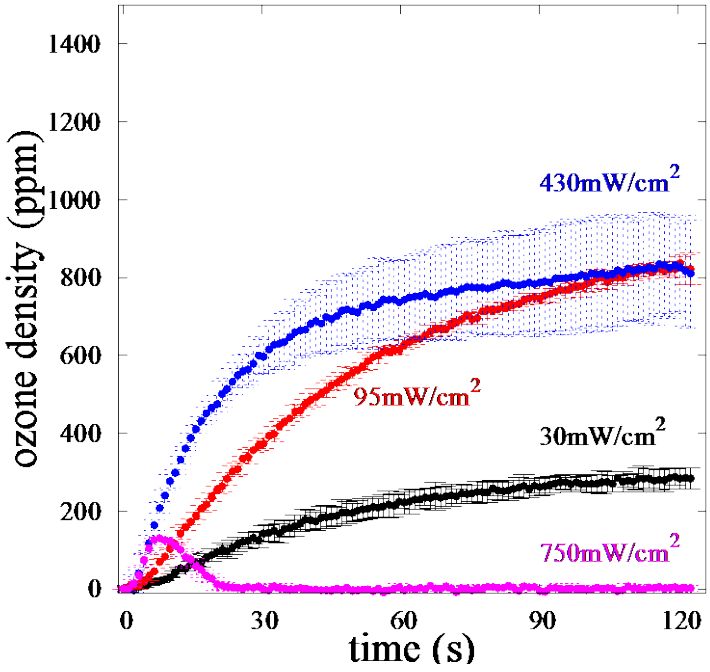


Figure 25: Ozone density for mode transition electrode at wet conditions. Until 430 mW/cm^2 input power, the ozone concentration increased monotonically. With 750 mW/cm² input power, there is a sudden drop in the ozone concentration after 15 s.

Figure 25 shows the ozone measurement for the mode transition electrode with a small amount of humidity (see 4.10). Until an input power of 430 mW/cm², the ozone concentration increases monotonically. At 750 mW/cm², there is a sudden drop after 15 seconds.

Bactericidal effect

The data (Figure 26) show that a treatment time of 30 s leads to a reduction of 5 log steps in the target area with power consumptions of 95, 430 and 750 mW/cm². 15 seconds of CAP application only leads to a 5 log reduction with a power consumption of 430 mW/cm². With 30 mW/cm² higher treatment times (at least 45 s) were necessary to obtain a countable reduction of CFUs. This reduction ranges between 0.5 and 1 log.

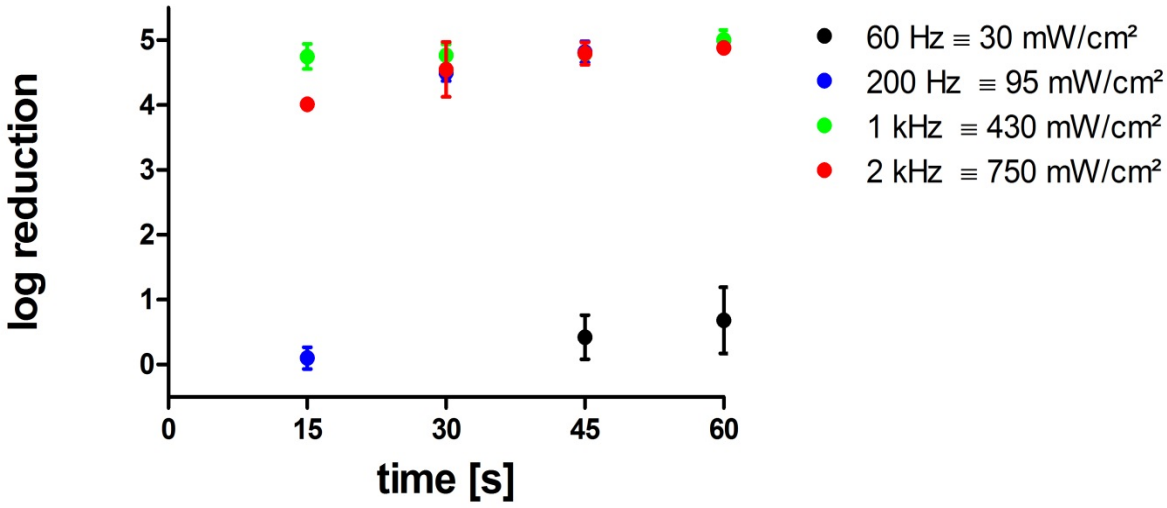


Figure 26: Bacterial reduction for different plasma treatment times and power consumptions. Applied voltage was always 8.5 kV_{PP} and the following applied frequencies were used to control the power input: 60 Hz, 200 Hz, 1 kHz, and 2 kHz. With power consumptions of 95, 430 and 750 mW/cm² a log 5 reduction could be achieved after 30 s.

Our experiments also show that oxidative stress evoked by ozone leads to bacterial inactivation (Figure 27) and that the ozone dose correlates with the log reduction of *E.coli*. Higher ozone density results in higher bacterial reduction.

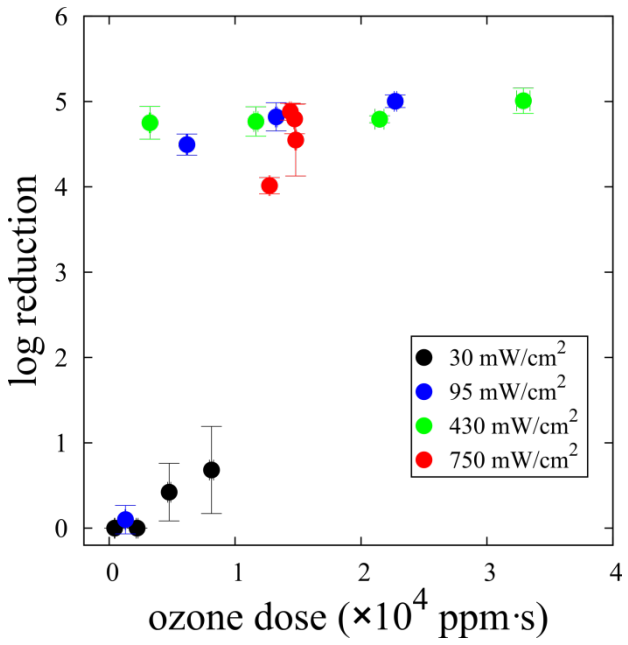


Figure 27: Ozone dose plotted against log reduction of bacteria. The ozone concentration produced by the mode transition electrode [ppm·s] at different power inputs correlates with the log reduction of *E.coli*. Higher ozone density results in higher bacterial reduction.

Viability of primary human dermal fibroblasts

As described above by applying different frequencies the amount of ozone that can be detected inside the closed volume varies. Aim of this viability test was to determine the impact of different concentrations of ozone on primary human dermal fibroblasts.

The results show that there is no big difference in the total cell numbers (Figure 28). One can discern a trend in decreasing cellnumbers towards higher treatment times

and increasing power consumptions when looking at a close up of the scale (Figure 28 b). Nevertheless the differences are statistically not significant.

a) b)

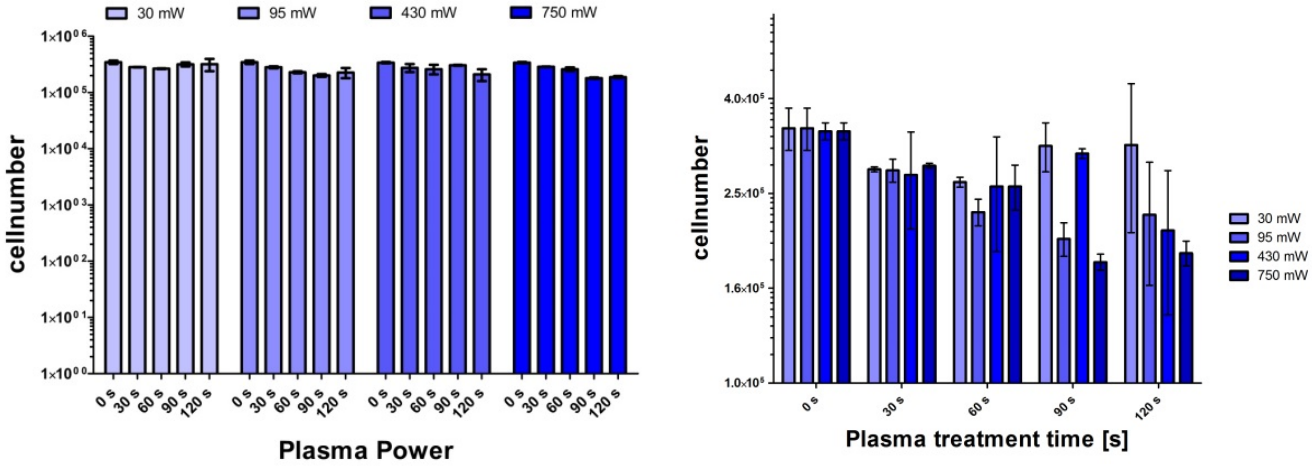


Figure 28: a) Cellnumbers 48 hours after plasma treatment. Cells were treated for 30 s, 60 s, 90 s and 120 s. Controls were untreated cells. Voltage was always 8.5 kV_{PP}. The cells were counted with a Hemocytometer. b) The same graph with a larger scale.

Figure 29 a shows the correlation between the ozone concentration produced by the plasma of the mode transition electrode and the cell viability of primary human dermal fibroblasts. In Figure 29 b the “therapeutic window” can be determined as the region, where a 5 log reduction of *E.coli* could be achieved and a cell viability of 75 % in dermal fibroblasts could be ensured.

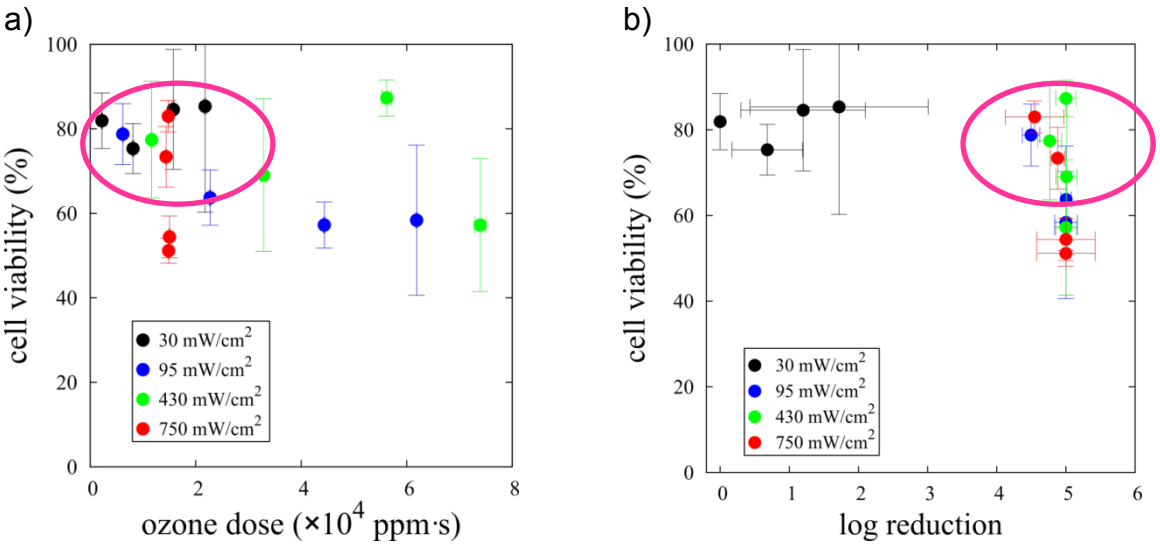


Figure 29:
a) Ozone dose plotted versus cell viability. With increasing ozone dose the cell viability of dermal fibroblasts decreases. A cell viability of ~75 % depicts an acceptable low proportion of harmed cells in agreement with medical doctors.
b) Cell viability plotted against bacterial log reduction. The "therapeutic window" is marked with the magenta circle. Here, there is the best overlap where bacteria are inactivated and cell viability is not under 75 %. Error bars are standard deviation.

The limiting ozone dose here is $2 \cdot 10^4$ ppm·s - the corresponding ozone flux is $1.4 \cdot 10^{22}$ O_3/cm^2:

$$D = n_{O_3} \cdot v \cdot t = (2 \cdot 10^{-2} \cdot 2.8 \cdot 10^{19}) \frac{O_3}{cm^3} \cdot 2.5 \cdot 10^4 \frac{cm}{s} \cdot s = 1.4 \cdot 10^{22} \, {}^{O_3}/_{cm^2}$$

Conclusion

Intention of the experiments with the mode transition electrode was to investigate the influence of different ozone concentrations on bacteria and on human cells. Taken as a whole, ozone is harmful for all kinds of cells. But our experiments demonstrate that prokaryotes, at least *E.coli*, are more disturbed by the higher ozone concentration than primary human dermal fibroblasts and that there is a window where bacteria are already inactivated and fibroblasts are still healthy.

5.4 HPRT assay (MiniFlatPlaSter)

Another aspect of safety is that no long term effects are caused by the treatment. As UV light in the range of 200 - 440 nm is emitted by the SMD devices the emergence of mutations has to be ruled out. One standard way of examination is the HPRT assay. To simulate a repeated treatment in clinics, not only single treatments were performed but also treatments every 12 hours.

Reference experiments with *E.coli*

To evaluate a reasonable duration of CAP-treatments for the mutagenicity tests, the reduction rate of 'wet' *E.coli* was measured. The data (Figure 30) show that a treatment time of 120 sec leads to a reduction of 5 log steps in the target area. A reduction of $>10^6$-fold was achieved when the treatment time with CAP was 240 sec. For treatment times shorter than 60 sec the number of surviving CFUs in the target area was too high for counting. For 'sufficiently dry' bacteria on agar (dried for 30 min after inoculation), a column '30 s dry' was added to Figure 30: in this case a CAP treatment time of 30 seconds led to a reduction of almost 10^5-fold.

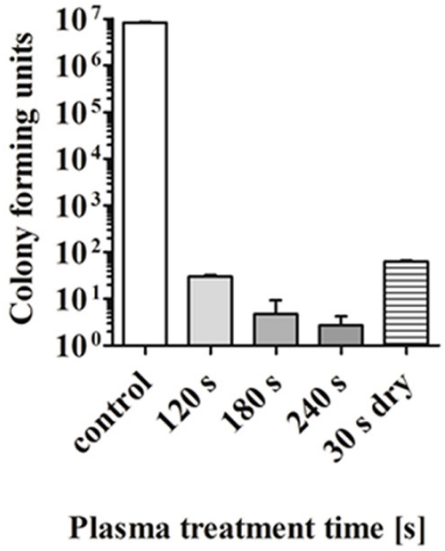


Figure 30: Survival of colony forming units (cfu) after different CAP treatment times. The control shows the initially plated number of cfu. The data for '120 s', '180 s' and '240 s' were obtained by treating the bacteria with CAP on agar, immediately after inoculation ('wet'). The data for '30 s dry' refer to the measurement on a 'dry' agar surface – where a drying time of 30 min was added after inoculation of the bacterial suspension. The error bars correspond to the standard deviation. [69]

Cell viability

For the HPRT test, the V79 cells were treated with CAP (for experimental setup see Figure 13) and afterwards cultured in medium for three days. The number of cells on day 4 is presented in Figure 31 and suggests that the number of cells treated for 240 sec with CAP is in the same range as the number of cells initially seeded (no significant difference, $P<0.001$). Cell numbers for treatment times up to 60 sec showed no significant difference compared with the untreated control ($P<0.001$).

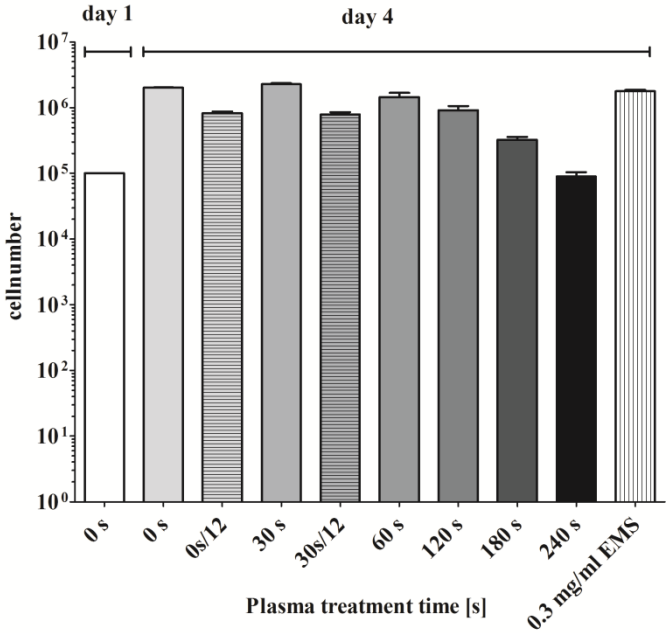


Figure 31: Counted cell numbers on day 1 and day 4 after different CAP treatment times, or application of EMS. The error bars show the standard deviations. [69]

Within the used plasma treatment times, both the cell counts and the microscope images of the cells (taken after CAP treatment, Figure 32) suggest a decelerated proliferation speed rather than apoptosis or necrosis. In microscope images showing V79 cells before, immediately after, and 1 hour, 18, 24, 48 and 72 hours after a CAP treatment of 240 sec duration, we could not find any evidence for cell death in the images (Figure 32). This was taken to support the following hypothesis that the cells stopped or slowed down their proliferation due to the CAP treatment.

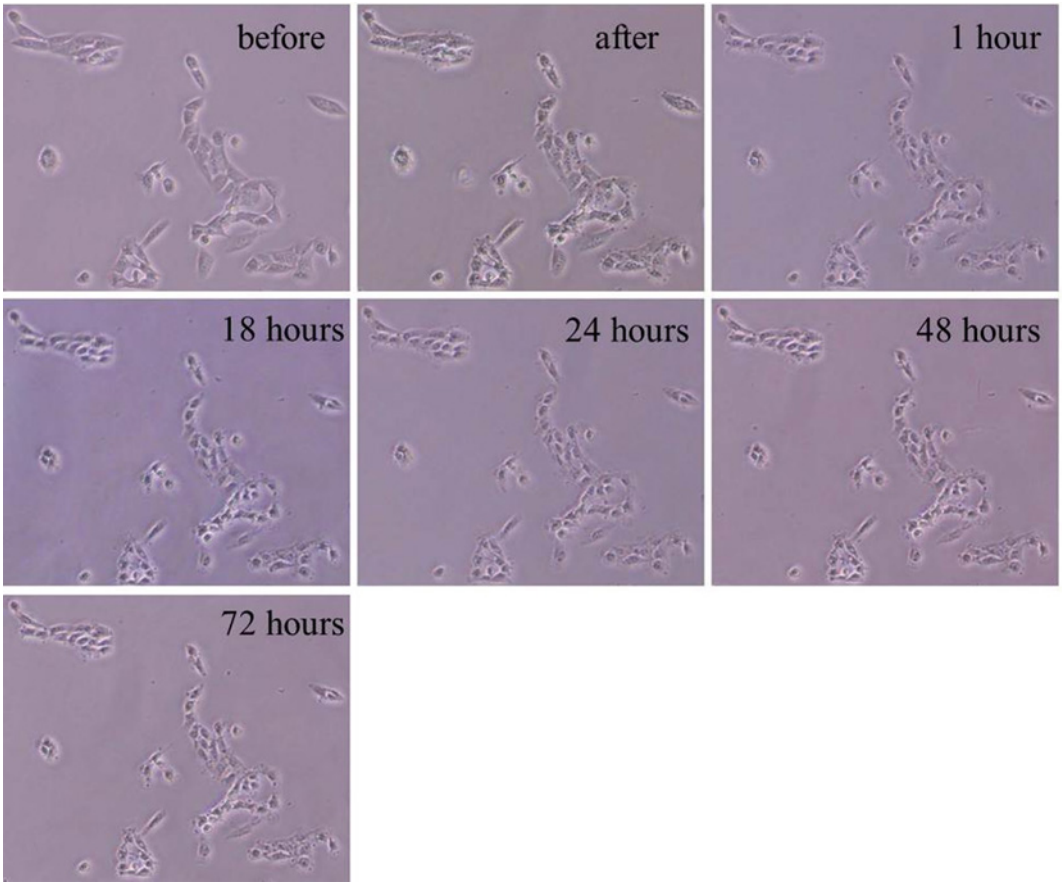


Figure 32: Representative microscope images (10 x magnification) showing V79 cells before, immediately after, and 1 hour, 18, 24, 48, 72 hours after the CAP treatment of 240 sec. Although the proliferation seems to be decelerated, no apoptotic or necrotic behaviour is visible. [69]

Mutant frequency

On day 4 cells were replated (after cell count) and medium was supplemented with 5 µg/ml of the selective agent 6-TG (Sigma Aldrich). Mutant colonies were counted after 8-10 days of incubation. The results of the mutagenicity test are presented in Figure 33. CAP treatments of up to 240 sec and repeated CAP treatments of 30 sec every 12 hours induced at maximum nine mutants per 10^5 cells. The control treatment with 0.3 mg/ml EMS for three hours induced up to 288 mutants per 10^5 cells as expected according to Bradley *et al*. [102-104] Corresponding to the

literature [102-106] and the control (untreated) samples, the mutant frequency observed for the CAP-treated samples lies in the range of the naturally occurring mutation rate of V79 cells. This means that CAP treatments of up to 240 sec did not cause mutations in V79 cells.

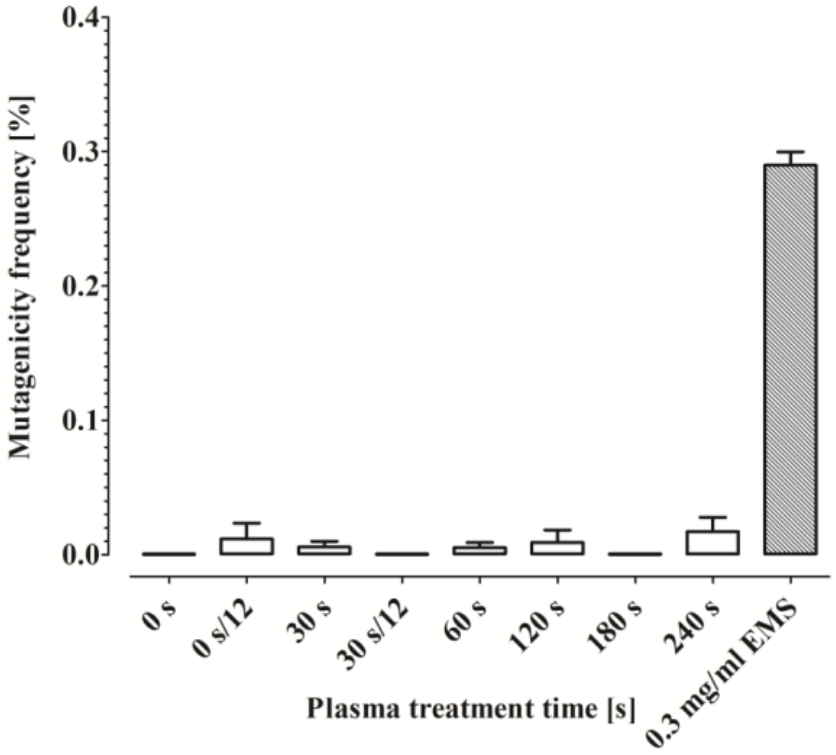


Figure 33: Mutant frequency for the negative controls, the EMS treatment and the different CAP treatment times. The initially seeded cell number was 10^5 V79 cells per well. The error bars show the standard deviations. [69]

Conclusion

The HPRT assay was performed with the MiniFlatPlaSter. But the amount of UV light and the range of the emission are comparable for all the SMD devices. The results of this study are therefore important for all further studies as it was shown that mutations due to CAP do not have to be expected.

6. Discussion

Impact of reactive species on bacterial inactivation by CAP

The ability to inactivate bacteria with cold atmospheric plasma was demonstrated by various groups worldwide and with a wide range of different kinds of pathogens [7, 27, 30, 92, 93, 107-112] and scrutinized within the first part of this study.

The inactivation of *E.coli* with CAP was therefore not new, nevertheless most publications and studies regarding this subject dealt with bacteria on agar, which is a rather dry surface. Wounds in patients represent a predominantly wet environment were bacteria can be hidden in the wound fluid. [113] The aim of the first part of this study was thus to investigate whether pathogens in solutions can also be inactivated by CAP. As mentioned earlier, the number of pathogens also plays an important role for wound healing so the experiments were additionally carried out with different initial cellnumbers in the fluid. The results demonstrate that *E.coli* can be inactivated within a short time but that there is a critical number of 10^7 per 20 µl above which no more cells are inactivated. To get a deeper insight in these effects the emergence of hydrogen peroxide and nitrites/nitrates into the liquid was measured. While the concentration of H_2O_2 seems to saturate, the amount of nitrites and nitrates increases steadily with increasing treatment times. The reference experiments with hydrogen peroxide and a chemical NO donor on the other side clearly demonstrate that the bactericidal property which was achieved after 3 min of CAP application could only be reached with significant higher dosages of H_2O_2 and NO.

Many publications in recent time dealt with “plasma activated water” which means that water is treated with CAP and then bacteria are inserted into this. [5, 52, 114] All groups could show that by increasing the mixing rate the bactericidal efficacy could be significantly increased. Increasing the mixing rate means for example shaking or stirring the liquid during CAP application. These results are promising as it shows that bacteria could be inactivated in even shorter time than shown in this study and consequently shorter treatment times are necessary.

Safety of CAP in primary culture

Different types of dermal cells and immune cells - such as keratinocytes and dermal fibroblasts - are incorporated in the proceeding of normal wound healing: hemostasis, coagulation and inflammation. [113] It begins with inflammation and immune cell recruitment to protect tissue from foreign invaders, such as bacteria and viruses. In the next phase the skin is resurfaced by stimulating regeneration and repair through proliferation of fibroblasts and re-epithelialization of keratinocytes. Wound healing plays an important role in protecting the human body from external infections. Cell migration and proliferation of dermal fibroblasts – amongst others - are therefore essential for proper wound healing. [115, 116]

As mentioned above, migration and proliferation of fibroblasts are crucial in wound healing. Cell lines that are used in cell cultures for all kinds of *in vitro* examinations are normally immortalized to provide easy handling and passaging. Strictly speaking this means certain ways of natural cell death were knocked out. In terms of safety of a medical device, in our case the SMD device, it is not very meaningful if

immortalized cells are not harmed by the treatment. Therefore it was necessary to maintain primary cells from fresh human specimen to investigate the behaviour of dermal fibroblasts after CAP application. Although an *in vitro* model always depicts an artificial environment, it is indispensable for all kinds of basic research.

To investigate the influence of CAP on migration and proliferation, primary human dermal fibroblasts were treated with CAP for certain times. The cellnumber was counted after 48 hours and scratch assays were performed and also observed for 48 hours. Results of these experiments show a trend towards a decline in cellnumbers with increasing treatment time. A deceleration in migration can also be detected with higher CAP dosage after 24 hours, but recovers afterwards. 48 hours after CAP treatment only cells that were treated for 120 s were not able to close the gap. The analysis of the cell cycle distribution supported the suggestion that cells are slowed down in proliferation. An arrest in G2 / M phase is visible for cells that were treated for 90 seconds and longer. This arrest was prominent 24 hours after CAP treatment and sustained for at least 72 hours.

To implement *in vitro* studies before bringing new devices to clinical studies is an essential prerequisite. Therefore the HPRT (hypoxanthine-guanine phosphoribosyl transferase) assay with V79 Chinese Hamster lung fibroblasts was carried out to investigate the mutagenic potential of CAP. Not only single treatment times, that were far above the intended application time of CAP, but also repeated plasma treatments were performed to simulate a clinical application cycle. As the bactericidal effect is already at its maximum after 30 seconds on agar and after 3 min in solution, the chosen treatment times of 240 seconds and 30 seconds every 12 hours

represent an adequate model for this. V 79 cells are the standard cell line to perform the HPRT assay as mutations can be induced easily. Control treatments with a mutagenic agent and with V79 cells that were not treated by plasma or any agent clearly show that CAP produced by the SMD device does not evoke mutations beyond naturally occurring spontaneous mutations in V79 cells.

This was an important finding as the MiniFlatPlaSter, a representative for CAP devices based on the SMD technology, now proved its bactericidal efficacy and the results of the HPRT assay paved the way to clinical studies.

Towards designed plasma and a “therapeutic window” – CAP and wound healing

During the first stage of wound repair – inflammation - microbes are inactivated via reactive oxygen species (among others). [117]

Most of the microorganisms have a limited tolerance for oxygen. This property is most evident in the case of obligate anaerobes and microaerophiles, which cannot grow in air saturated media, but it is also true for committed aerobes, which grow poorly or die when they are exposed to hyperoxia. [53] Some immune cells which use the NADPH oxidase enzyme, upon invasion by pathogenic bacteria, also exploit oxidative stress as a weapon during phagocytosis. And recently evidence was reported that the bactericidal effects of antibiotics with different sites of action rely, in part, on the elevation in ROS that they elicit. [118]

The second stage of wound repair — new tissue formation — occurs 2–10 days after injury and is characterized by cellular proliferation and migration of different cell types. [117, 119] These immune cells and all cell types that are involved in wound healing - platelets, inflammatory cells, fibroblasts and epithelial cells - are also capable of producing nitric oxide (NO), either constitutively or in response to inflammatory cytokines. [61, 120] Among its many effects, NO plays a critical role in host defence by clearing the wound of infectious microbes and stimulating other healing factors to access the wound area by maintaining a constant blood flow. [60]

Various groups are working with methods to deliver NO to chronic wounds to improve wound healing and accelerate wound closure. The antimicrobial property of NO is also notable in this context which obviously can also be of interest for wound

healing. [121, 122] However, the question regarding the optimal dose needed for these different modes of action still remains unanswered. Also application forms, from topical application of ointments to gaseous NO, are tested. [57, 121, 123, 124]

But nevertheless the dominant element that seems to be responsible for the bactericidal effect of CAP is oxygen or rather reactive oxygen species. [35] The cold atmospheric plasma device that was used for the examination of the safety of CAP on primary fibroblasts was operated mainly in the ozone based mode (Figure 11).

Dermal fibroblasts were slowed down in their migration speed and partially inhibited in proliferation. But the analysis of the cell cycle distribution and long-time observation of the migration showed that those effects only persist for treatment times above 90 s.

Other studies with CAP also dealt with migration and cell cycle distribution. Although subject of those investigations were mostly cancer cells, it is noticeable that here the decrease in migration speed as well as a G2 / M phase arrest were much more dominant, already with lower plasma doses than in our study. [125-127] Also the persistence of the arrest was up to several days. [125]

Arndt et al. [128] could even show improved wound healing *in vitro* and *in vivo* on a mouse model with punch biopsies. Additionally they suggest senescence as a probable effect of CAP on melanoma cells [129] which could be a possible hint towards selectivity of CAP. Also important and already shown by for example Daeschlein et al. [130] is that CAP is well tolerated by skin and does not harm the natural skin flora. Not in the focus of the study but a passing reference of our study

with two different handheld devices was that volunteers had no negative side effects after the plasma application of their arms or the skin respectively. [131]

All observed effects – on prokaryotes as well as on eukaryotes - were enhanced by increasing plasma dose or treatment time respectively (for example [41, 77, 109, 132-134]). But microorganisms seem to be much more susceptible towards the reactive species produced by cold atmospheric plasma than normal human cells which we could show in our own studies (Figure 27 and Figure 28) and what can be seen in literature. [6, 10, 15, 135] The decisive point is to find the “therapeutic window” where pathogens are already inactivated and normal human cells are not yet harmed. Table 5 gives a short summary of all studies that were carried out with SMD devices to investigate the bactericidal efficacy as well as the safety of CAP application on human skin.

Table 5: Results of different studies with different SMD devices to assess the bactericidal efficacy and the safety upon dermal cells.

Power consumption	Treatment time	Summary
20 mW/cm²	20 seconds	*D. radiodurans* and *MRSA* could be reduced 5 log steps by applying CAP for 20 seconds with the FlatPlaSter 2.0.
20 mW/cm²	90 seconds	Results of this study show that there is no long term damage to primary human dermal fibroblasts when treated with the FlatPlaSter 2.0 for up to 90 seconds.
35 mW/cm²	15 seconds 30 seconds	Treatment of *S.aureus* with the FlatPlaSter 2.0 on agar resulted in a 5.5 log reduction after 15 seconds. After 30 seconds a 5 log reduction of MRSA and many other microorganisms of clinical interest was achieved. [7]
20 mW/cm² **500 mW/cm²**	20 minutes	HE staining of histological samples of plasma treated ex vivo skin with FlatPlaSter 2.0 and MiniFlatPlaSter showed no damage after treatment times up to 20 minutes. [14]
500 mW/cm²	30 seconds	Human skin on the forearm of volunteers was inoculated with bacteria and treated with two different handheld plasma devices (direct and indirect electrodes). After 30 seconds a 2.5 log reduction was achieved and no visible damage to the skin could be detected. [131]
500 mW/cm²	60 seconds	Immunohistochemical γ-H2AX staining of the ex vivo skin samples showed that plasma treatments up to 60 s could be considered as a safe application time using MiniFlatPlaSter. [14]
500 mW/cm²	60 seconds	A bacterial reduction of up to 3 log steps was achieved after 60 seconds of plasma treatment of *in vitro* porcine skin samples inoculated with bacteria. [6]
500 mW/cm²	240 seconds	The HPRT assay with V79 Chinese hamster lung fibroblasts revealed no mutagenicity after treatment with the MiniFlatPlaSter for treatment times up to 240 seconds. [69]
500 mW/cm²	15 minutes	The TUNEL staining of skin sections after plasma treatment of skin samples inoculated with bacteria revealed no damage to intact porcine skin *in vitro*. [6]

The shift from mainly ozone (generated at low power input) to nitrogen oxides (produced at high power input) that can be observed is a first step towards “designed” SMD plasmas dependent to their field of application - wound healing, sterilization or cancer treatment. Reactive oxygen species are bactericidal, but: by adjusting the SMD plasma at certain power levels ozone will be quenched and converted into nitrogen oxides as described above. [35]

Nitrogen oxides will be further converted to nitric acid or nitrous acid in ambient air when trace amount of water vapour is present. In return these species could then serve as a source for NO molecules, which are known to stimulate wound healing. [57, 59, 60, 121, 123, 124, 136, 137] Studies with nitric oxide-releasing nanoparticles already demonstrated accelerated wound healing by promoting fibroblast migration and collagen deposition. [59]

Therefore it is possible to find the exact “therapeutic window” depending on the application purpose (Figure 34).

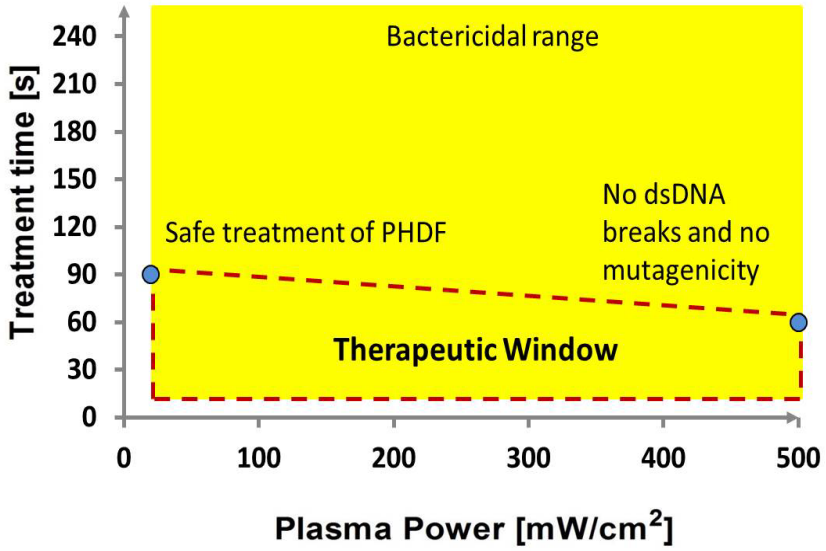


Figure 34: The graph shows the power settings and treatment times of several experiments that were performed with different SMD devices. The yellow area marks the antimicrobial field and the red circuit marks the area where dermal cells and skin are still healthy, a so called "therapeutic window".

In the example of wounds it would be imaginable to apply plasma with higher power first to reduce the bacterial load (the produced ozone can therefore inactivate the exposed bacteria within a few seconds) followed by treatment with SMD plasma ignited for power consumptions around 0.2 W/cm^2 in a confined volume to support wound healing and accelerate wound closure.

7. Outlook

Bacteria and other pathogens do not only disturb wound healing, they are responsible for other skin diseases as well. A few of them were already part of studies with CAP.

Positive track records could already be achieved in the therapy of herpes zoster. Herpes zoster is an acute skin disease due to varicella-zoster virus, a neurotropic human alpha-herpes virus and widespread in the population; the global median incidence of zoster is estimated to be 4 – 4.5 per 1000 person years, a lifetime risk of 30%. Incidence and severity rises with increasing age. Among persons older than 80 years, the incidence is 12.0 per 1000 years. [138, 139] First clinical trials with CAP treatment relieved acute pain and accelerated healing. [140] Single case reports give a hint that there are many of diseases where CAP could be an option. CAP was able to control local infection and reduce pain in a patient with chronic post-operative ear infection and lesions in Hailey-Hailey disease were successfully treated. [141, 142]

Dentistry is also a very promising field for the application of CAP because biofilms of for example *S. mutans*, which play a major role in the pathogenesis of peri-implant mucositis [143] have been successfully removed by different non-thermal plasma devices. This is also very promising for normal dental hygiene.

There are many future application fields for CAP – sooner or later. The possible treatment of cancer is already envisioned. First studies and mouse models showed promising results. [33, 38, 41, 125, 144, 145] And also in this field, designing the plasma is crucial. Here the “therapeutic window” depicts a frame where cancer cells

can selectively be ablated and the normal surrounding tissue remains unaffected. First attempts into this direction were made. [134]

Nevertheless until CAP will be implemented in clinical applications on human tumours is reality, a long way lies ahead. What seems to lie in near future is to disinfect wounds or surfaces. All work that was done in this study clearly points the way to the future where cold atmospheric plasma could be a medical tool in many fields, wound healing as one of them.

8. References

1. Laroussi, M., I. Alexeff, and W.L. Kang, *Biological decontamination by nonthermal plasmas.* Plasma Science, IEEE Transactions on, 2000. **28**(1): p. 184-188.

2. Shimizu, T., et al., *Characterization of microwave plasma torch for decontamination.* Plasma Processes and Polymers, 2008. **5**(6): p. 577-582.

3. Deng, X.T., et al., *Effects of microbial loading and sporulation temperature on atmospheric plasma inactivation of Bacillus subtilis spores.* Applied Physics Letters, 2005. **87**(15): p. 153901.

4. Stoffels, E., Y. Sakiyama, and D.B. Graves, *Cold Atmospheric Plasma: Charged Species and Their Interactions With Cells and Tissues.* Plasma Science, IEEE Transactions on, 2008. **36**(4): p. 1441-1457.

5. Traylor, M.J., et al., *Long-term antibacterial efficacy of air plasma-activated water.* Journal of Physics D-Applied Physics, 2011. **44**(47): p. 472001.

6. Maisch, T., et al., *Decolonisation of MRSA, S. aureus and E. coli by Cold-Atmospheric Plasma Using a Porcine Skin Model In Vitro.* PLoS One, 2012. **7**(4): p. e34610.

7. Klampfl, T.G., et al., *Cold Atmospheric Air Plasma Sterilization against Spores and Other Microorganisms of Clinical Interest.* Applied and environmental microbiology, 2012. **78**(15): p. 5077-82.

8. Weltmann, K.D. and et al., *Atmospheric Pressure Plasma Jet for Medical Therapy: Plasma Parameters and Risk Estimation.* Contributions to Plasma Physics, 2009. **49**(9): p. 631.

9. Hammann, A., et al., *Antiseptic efficacy and tolerance of tissue-tolerable plasma compared with two wound antiseptics on artificially bacterially contaminated eyes from commercially slaughtered pigs.* Skin Pharmacol Physiol, 2010. **23**(6): p. 328-32.

10. Lademann, O., et al., *Skin Disinfection by Plasma-Tissue Interaction: Comparison of the Effectivity of Tissue-Tolerable Plasma and a Standard Antiseptic.* Skin Pharmacology and Physiology, 2011. **24**(5): p. 284-288.

11. Zimmermann, J.L., et al., *Test for bacterial resistance build-up against plasma treatment.* New Journal of Physics, 2012. **14**(7): p. 073037.

12. Matthes, R., O. Assadian, and A. Kramer, *Repeated applications of cold atmospheric pressure plasma does not induce resistance in Staphylococcus aureus embedded in biofilms.* GMS Hyg Infect Control, 2014. **9**(3): p. Doc17.

13. Isbary, G., et al., *A first prospective randomized controlled trial to decrease bacterial load using cold atmospheric argon plasma on chronic wounds in patients.* British Journal of Dermatology, 2010. **163**(1): p. 78-82.

14. Isbary, G., et al., *Ex vivo human skin experiments for the evaluation of safety of new cold atmospheric plasma devices.* Clinical Plasma Medicine, 2013. **1**(1): p. 36-44.

15. Lademann, O., et al., *Stimulation of the penetration of particles into the skin by plasma tissue interaction.* Laser Physics Letters, 2011. **8**(10): p. 758-764.

16. Kramer, A., et al., *Suitability of tissue tolerable plasmas (TTP) for the management of chronic wounds.* Clinical Plasma Medicine, 2013. **1**(1): p. 11-18.

17. Langmuir, I., *Modern Concepts in Physics and Their Relation to Chemistry.* Science, 1929. **70**(1817): p. 385-96.

18. Fridman, A., *Plasma Chemistry*. 2008, Cambridge University Press: New York.

19. Nosenko, T., T. Shimizu, and G.E. Morfill, *Designing plasmas for chronic wound disinfection.* New Journal of Physics, 2009. **11**(11): p. 115013.

20. Morfill, G.E., et al., *Nosocomial infections-a new approach towards preventive medicine using plasmas.* New Journal of Physics, 2009. **11**(11): p. 115019.

21. Fridman, G., et al., *Blood Coagulation and Living Tissue Sterilization by Floating-Electrode Dielectric Barrier Discharge in Air.* Plasma Chemistry and Plasma Processing, 2006. **26**(4): p. 425-442.

22. Ermolaeva, S.A., et al., *Bactericidal effects of non-thermal argon plasma in vitro, in biofilms and in the animal model of infected wounds.* Journal of Medical Microbiology, 2011. **60**(1): p. 75-83.

23. Shashurin, A., et al., *Influence of Cold Plasma Atmospheric Jet on Surface Integrin Expression of Living Cells.* Plasma Processes and Polymers, 2010. **7**(3-4): p. 294-300.

24. Vandamme, M., et al., *Antitumor Effect of Plasma Treatment on U87 Glioma Xenografts: Preliminary Results.* Plasma Processes and Polymers, 2010. **7**(3-4): p. 264-273.

25. Lu, X.P., et al., *A single electrode room-temperature plasma jet device for biomedical applications.* Applied Physics Letters, 2008. **92**(15).

26. Foest, R., et al., *Non-thermal atmospheric pressure discharges for surface modification.* Plasma Physics and Controlled Fusion, 2005. **47**: p. B525-B536.

27. Maisch, T., et al., *Contact-free cold atmospheric plasma treatment of Deinococcus radiodurans.* Journal of industrial microbiology & biotechnology, 2012. **39**(9): p. 1367-75.

28. Jarrige, J., M. Laroussi, and E. Karakas, *Formation and dynamics of plasma bullets in a non-thermal plasma jet: influence of the high-voltage parameters on the plume characteristics.* Plasma Sources Science & Technology, 2010. **19**(6).

29. Pei, X., et al., *Inactivation of a 25.5 μm Enterococcus faecalis biofilm by a room-temperature, battery-operated, handheld air plasma jet.* Journal of Physics D: Applied Physics, 2012. **45**(16): p. 165205.

30. Sladek, R.E.J. and E. Stoffels, *Deactivation of Escherichia coli by the plasma needle.* Journal of Physics D-Applied Physics, 2005. **38**(11): p. 1716-1721.

31. Iseki, S., et al., *Rapid inactivation of Penicillium digitatum spores using high-density nonequilibrium atmospheric pressure plasma.* Applied Physics Letters, 2010. **96**(15): p. 153704.

32. von Woedtke, T., et al., *Plasmas for medicine.* Physics Reports-Review Section of Physics Letters, 2013. **530**(4): p. 291-320.

33. Schlegel, J., J. Köritzer, and V. Boxhammer, *Plasma in cancer treatment.* Clinical Plasma Medicine, 2013. **1**(2): p. 2-7.

34. Pavlovich, M.J., et al., *Effect of Discharge Parameters and Surface Characteristics on Ambient-Gas Plasma Disinfection.* Plasma Processes and Polymers, 2013. **10**(1): p. 69-76.

35. Shimizu, T., et al., *The dynamics of ozone generation and mode transition in air surface micro-discharge plasma at atmospheric pressure.* New Journal of Physics, 2012. **14**.

36. Sakiyama, Y., et al., *Plasma chemistry model of surface microdischarge in humid air and dynamics of reactive neutral species.* Journal of Physics D: Applied Physics, 2012. **45**(42): p. 425201.

37. Sakiyama, Y., et al., *Finite element analysis of ring-shaped emission profile in plasma bullet.* Applied Physics Letters, 2010. **96**(4).

38. Vandamme, M., et al., *Response of Human Glioma U87 Xenografted on Mice to Non Thermal Plasma Treatment.* Plasma Medicine, 2011. **1**(1): p. 27-43.

39. Bundscherer, L., et al., *Impact of non-thermal plasma treatment on MAPK signaling pathways of human immune cell lines.* Immunobiology, 2013.

40. Lademann, J., et al., *Risk assessment of the application of tissue-tolerable plasma on human skin.* Clinical Plasma Medicine, 2013. **1**(1): p. 5-10.

41. Barekzi, N. and M. Laroussi, *Dose-dependent killing of leukemia cells by low-temperature plasma.* Journal of Physics D-Applied Physics, 2012. **45**(42).

42. Isbary, G., et al., *Successful and Safe Use of 2 Min Cold Atmospheric Argon Plasma in Chronic Wounds: Results of A Randomized Controlled Trial.* British Journal of Dermatology, 2012. **167**(2): p. 404-10.

43. Hoentsch, M., et al., *Time-dependent effects of low-temperature atmospheric-pressure argon plasma on epithelial cell attachment, viability and tight junction formation in vitro.* Journal of Physics D: Applied Physics, 2012. **45**(2): p. 025206.

44. Morfill, G.E., M.G. Kong, and J.L. Zimmermann, *Focus on Plasma Medicine.* New Journal of Physics, 2009. **11**: p. 115011.

45. Shimizu, T., J.L. Zimmermann, and G.E. Morfill, *The bactericidal effect of surface micro-discharge plasma under different ambient conditions.* New Journal of Physics, 2011. **13**(2): p. 023026.

46. Staehelin, J., R.E. Buehler, and J. Hoigne, *Ozone decomposition in water studied by pulse radiolysis. 2. Hydroxyl and hydrogen tetroxide (HO4) as chain intermediates.* The Journal of Physical Chemistry, 1984. **88**(24): p. 5999-6004.

47. Treinin, A. and E. Hayon, *Absorption spectra and reaction kinetics of NO2, N2O3, and N2O4 in aqueous solution.* Journal of the American Chemical Society, 1970. **92**(20): p. 5821-5828.

48. Fischer, M. and P. Warneck, *Photodecomposition of Nitrite and Undissociated Nitrous Acid in Aqueous Solution.* The Journal of Physical Chemistry, 1996. **100**(48): p. 18749-18756.

49. Zafiriou, O.C. and R. Bonneau, *WAVELENGTH-DEPENDENT QUANTUM YIELD OF OH RADICAL FORMATION FROM PHOTOLYSIS OF NITRITE ION IN WATER.* Photochemistry and Photobiology, 1987. **45**: p. 723-727.

50. Graves, D.B., *The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology.* Journal of Physics D: Applied Physics, 2012. **45**(26): p. 263001.

51. Rubbo, H., et al., *Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives.* J Biol Chem, 1994. **269**(42): p. 26066-75.

52. Pavlovich, M.J., et al., *Ozone correlates with antibacterial effects from indirect air dielectric barrier discharge treatment of water.* Journal of Physics D: Applied Physics, 2013. **46**(14): p. 145202.

53. Imlay, J.A., *Pathways of oxidative damage.* Annu Rev Microbiol, 2003. **57**: p. 395-418.

54. Martindale, J.L. and N.J. Holbrook, *Cellular response to oxidative stress: Signaling for suicide and survival*.* Journal of Cellular Physiology, 2002. **192**(1): p. 1-15.

55. Lineaweaver, W., et al., *Cellular and bacterial toxicities of topical antimicrobials.* Plast Reconstr Surg, 1985. **75**(3): p. 394-6.

56. Hogg, N. and B. Kalyanaraman, *Nitric oxide and lipid peroxidation.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1999. **1411**(2–3): p. 378-384.

57. Bernatchez, S.F., et al., *Nitric oxide levels in wound fluid may reflect the healing trajectory.* Wound Repair and Regeneration, 2013. **21**(3): p. 410-417.

58. Darley-Usmar, V.M., et al., *Antioxidant Actions of Nitric Oxide*, in *Nitric Oxide*, J.I. Louis, Editor. 2000, Academic Press: San Diego. p. 265-276.

59. Han, G., et al., *Nitric Oxide–Releasing Nanoparticles Accelerate Wound Healing by Promoting Fibroblast Migration and Collagen Deposition.* The American Journal of Pathology, 2012. **180**(4): p. 1465-1473.

60. Schaffer, M.R., et al., *Nitric oxide regulates wound healing.* J Surg Res, 1996. **63**(1): p. 237-40.

61. Schwentker, A., et al., *Nitric oxide and wound repair: role of cytokines?* Nitric Oxide, 2002. **7**(1): p. 1-10.

62. Weller, R., *Nitric oxide--a newly discovered chemical transmitter in human skin.* Br J Dermatol, 1997. **137**(5): p. 665-72.

63. Weller, R., et al., *Nitric oxide is generated on the skin surface by reduction of sweat nitrate.* J Invest Dermatol, 1996. **107**(3): p. 327-31.

64. Weller, R., *Nitric oxide donors and the skin: useful therapeutic agents?* Clin Sci (Lond), 2003. **105**(5): p. 533-5.

65. Weller, R., *Nitric oxide, skin growth and differentiation: more questions than answers?* Clin Exp Dermatol, 1999. **24**(5): p. 388-91.

66. Boxhammer, V., et al., *Bactericidal action of cold atmospheric plasma in solution.* New Journal of Physics, 2012. **14**(11): p. 113042.

67. Kogelschatz, U., *Dielectric-Barrier Discharges: Their History, Discharge Physics, and Industrial Applications.* Plasma Chemistry and Plasma Processing, 2003. **23**(1): p. 1-46.

68. Li, Y.-F., J.L. Zimmermann, and G.E. Morfill, *Optimizing the distance for bacterial treatment using surface micro-discharge plasma.* New Journal of Physics, 2012. **14**(2): p. 023058.

69. Boxhammer, V., et al., *Investigation of the mutagenic potential of cold atmospheric plasma at bactericidal dosages.* Mutat Res, 2013.

70. Casewell, M.W. and R.L. Hill, *The carrier state: methicillin-resistant Staphylococcus aureus.* The Journal of antimicrobial chemotherapy, 1986. **18 Suppl A**: p. 1-12.

71. *Annual Epidemiological Report On Communicable Diseases In Europe 2009*. 2009, European Centre for Disease Prevention and Control: Stockholm.

72. Wisplinghoff, H., et al., *Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. (vol 39, pg 309, 2004).* Clinical Infectious Diseases, 2005. **40**(7): p. 1077-1077.

73. Richards, M.J., et al., *Nosocomial infections in pediatric intensive care units in the United States. National Nosocomial Infections Surveillance System.* Pediatrics, 1999. **103**(4): p. e39.

74. Lowy, F.D., *Antimicrobial resistance: the example of Staphylococcus aureus.* J Clin Invest, 2003. **111**(9): p. 1265-73.

75. Chambers, H.F., *The changing epidemiology of Staphylococcus aureus?* Emerging infectious diseases, 2001. **7**(2): p. 178-82.

76. WHO, *ANTIMICROBIAL RESISTANCE Global Report on surveillance* 2014.

77. Bouza, E., et al., *A European perspective on intravascular catheter-related infections: report on the microbiology workload, aetiology and antimicrobial susceptibility (ESGNI-005 Study).* Clin Microbiol Infect, 2004. **10**(9): p. 838-42.

78. Cardo, D., et al., *National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004.* American journal of infection control, 2004. **32**(8): p. 470-485.

79. Klevens, R.M., et al., *Changes in the epidemiology of methicillin-resistant Staphylococcus aureus in intensive care units in US hospitals, 1992-2003.* Clinical Infectious Diseases, 2006. **42**(3): p. 389-391.

80. Carleton, H.A., et al., *Community-adapted methicillin-resistant Staphylococcus aureus (MRSA): Population dynamics of an expanding community reservoir of MRSA.* Journal of Infectious Diseases, 2004. **190**(10): p. 1730-1738.

81. King, M.D., et al., *Emergence of community-acquired methicillin-resistant Staphylococcus aureus USA 300 clone as the predominant cause of skin and soft-tissue infections.* Annals of internal medicine, 2006. **144**(5): p. 309-17.

82. Wise, R., *The urgent need for new antibacterial agents.* J Antimicrob Chemother, 2011. **66**(9): p. 1939-40.

83. Piddock, L.J.V., *The crisis of no new antibiotics—what is the way forward?* Lancet Infect Dis, 2012. **12**: p. 249-53.

84. Costelloe, C., et al., *Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis.* Bmj, 2010. **340**: p. c2096.

85. Pechere, J.C., *Patients' interviews and misuse of antibiotics.* Clin Infect Dis, 2001. **33 Suppl 3**: p. S170-3.

86. Canton, R. and M.I. Morosini, *Emergence and spread of antibiotic resistance following exposure to antibiotics.* FEMS Microbiol Rev, 2011. **35**(5): p. 977-91.

87. Carlet, J., et al., *Society's failure to protect a precious resource: antibiotics.* Lancet, 2011. **378**: p. 369-371.

88. Sacks, R. and L. Greene, *New management of gonorrhoea. Impact of antibiotic resistance.* BMJ, 2011. **342**: p. d3523.

89. Boyce, J.M. and D. Pittet, *Guideline for Hand Hygiene in Health-Care Settings. Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HIPAC/SHEA/APIC/IDSA Hand Hygiene Task Force.* American journal of infection control, 2002. **30**(8): p. S1-46.

90. *WHO Guidelines and Hand Hygiene in Health care*. Available from: http://www.who.int/patientsafety/safesurgery/en/.

91. *Liste der vom Robert Koch-Institut geprüften und anerkannten Desinfektionsmittel und -verfahren*, in *Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz* R. Koch-Institut, Editor. 2013, Springer-Verlag p. 1706-1728.

92. Maisch, T., et al., *Contact-free inactivation of Candida albicans biofilms by cold atmospheric air plasma.* Appl Environ Microbiol, 2012. **78**(12): p. 4242-7.

93. Koban, I., et al., *Treatment of Candida albicans biofilms with low-temperature plasma induced by dielectric barrier discharge and atmospheric pressure plasma jet.* New Journal of Physics, 2010. **12**: p. 073039.

94. Hähnel, M., T. von Woedtke, and K.-D. Weltmann, *Influence of the Air Humidity on the Reduction of Bacillus Spores in a Defined Environment at Atmospheric Pressure Using a Dielectric Barrier Surface Discharge.* Plasma Processes and Polymers, 2010. **7**(3-4): p. 244-249.

95. Laroussi, M., D.A. Mendis, and M. Rosenberg, *Plasma interaction with microbes.* New Journal of Physics, 2003. **5**: p. 341.

96. Fridman, G., et al., *Comparison of direct and indirect effects of non-thermal atmospheric-pressure plasma on bacteria.* Plasma Processes and Polymers, 2007. **4**(4): p. 370-375.

97. Robson, M.C., et al., *Maintenance of wound bacterial balance.* American journal of surgery, 1999. **178**(5): p. 399-402.

98. Robson, M.C., *Wound infection. A failure of wound healing caused by an imbalance of bacteria.* The Surgical clinics of North America, 1997. **77**(3): p. 637-50.

99. Fuchs, G., ed. *Allgemeine Mikrobiologie*. ed. H.G. Schlegel. Vol. 8. 2006, Georg Thieme Verlag: Stuttgart - New York.

100. Schmitz, S., ed. *Zellkultur*. Der Experimentator. 2009, Spektrum Akademischer Verlag: Heidelberg.

101. Davies, M.J., et al., *Molecular analysis of mutation at the hprt locus of Chinese hamster V79 cells induced by ethyl methanesulphonate and mitomycin C.* Mutation Research/Environmental Mutagenesis and Related Subjects, 1993. **291**(2): p. 117-124.

102. Bradley, M.O., et al., *Mutagenesis by chemical agents in V79 Chinese hamster cells: A review and analysis of the literature: A report of the gene-tox program.* Mutation Research/Reviews in Genetic Toxicology, 1981. **87**(2): p. 81-142.

103. Aaron, C.S., et al., *Molecular dosimetry of the chemical mutagen ethyl methanesulfonate: Quantitative comparison of mutation induction in Escherihia coli, V79 Chinese hamster cells and L5178Y mouse lymphoma cells, and some cytological results in vitro and in vivo.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1980. **69**(2): p. 201-216.

104. Chu, E.H. and H.V. Malling, *Mammalian cell genetics. II. Chemical induction of specific locus mutations in Chinese hamster cells in vitro.* Proceedings of the National Academy of Sciences, 1968. **61**(4): p. 1306-1312.

105. Aaron, C.S., et al., *Molecular dosimetry of the chemical mutagen ethyl methanesulfonate in Escherichia coli and in V-79 chinese hamster cells.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1978. **50**(3): p. 419-426.

106. Van Zeeland, A.A. and J.W.I.M. Simons, *Linear dose —response relationships after prolonged expression times in V-79 Chinese hamster cells.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1976. **35**(1): p. 129-137.

107. Sladek, R.E.J., et al., *Treatment of Streptococcus mutans biofilms with a nonthermal atmospheric plasma.* Letters in Applied Microbiology, 2007. **45**(3): p. 318-323.

108. Joshi, S.G., et al., *Control of methicillin-resistant Staphylococcus aureus in planktonic form and biofilms: a biocidal efficacy study of nonthermal dielectric-barrier discharge plasma.* American journal of infection control, 2010. **38**(4): p. 293-301.

109. Joshi, S.G., et al., *Nonthermal Dielectric-Barrier Discharge Plasma-Induced Inactivation Involves Oxidative DNA Damage and Membrane Lipid Peroxidation in Escherichia coli.* Antimicrobial agents and chemotherapy, 2011. **55**(3): p. 1053-62.

110. Schnabel, U., et al., *Decontamination of Microbiologically Contaminated Specimen by Direct and Indirect Plasma Treatment.* Plasma Processes and Polymers, 2012. **9**(6): p. 569-575.

111. Daeschlein, G., et al., *In Vitro Susceptibility of Important Skin and Wound Pathogens Against Low Temperature Atmospheric Pressure Plasma Jet (APPJ) and Dielectric Barrier Discharge Plasma (DBD).* Plasma Processes and Polymers, 2012. **9**(4): p. 380-389.

112. Daeschlein, G., et al., *Skin decontamination by low-temperature atmospheric pressure plasma jet and dielectric barrier discharge plasma.* Journal of Hospital Infection, 2012. **81**(3): p. 177-183.

113. Lazarus, G.S., et al., *Definitions and guidelines for assessment of wounds and evaluation of healing.* Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society, 1994. **2**(3): p. 165-70.

114. Oehmigen, K., et al., *The Role of Acidification for Antimicrobial Activity of Atmospheric Pressure Plasma in Liquids.* Plasma Processes and Polymers, 2010. **7**(3-4): p. 250-257.

115. Ho, M., et al., *Effects of the Novel Compound DK223 ([1E,2E-1,2-Bis(6-methoxy-2H-chromen-3-yl)methylene]hydrazine) on Migration and Proliferation of Human Keratinocytes and Primary Dermal Fibroblasts.* International Journal of Molecular Sciences, 2014. **15**(7): p. 13091-13110.

116. Gurtner, G.C., et al., *Wound repair and regeneration.* Nature, 2008. **453**(7193): p. 314-321.

117. Grose, R. and S. Werner, *Wound-healing studies in transgenic and knockout mice.* Molecular Biotechnology, 2004. **28**(2): p. 147-166.

118. Kohanski, M.A., et al., *A common mechanism of cellular death induced by bactericidal antibiotics.* Cell, 2007. **130**(5): p. 797-810.

119. WERNER, S. and R. GROSE, *Regulation of Wound Healing by Growth Factors and Cytokines*. Vol. 83. 2003. 835-870.

120. Bogdan, C., M. Rollinghoff, and A. Diefenbach, *Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity.* Curr Opin Immunol, 2000. **12**(1): p. 64-76.

121. Ghaffari, A., et al., *Potential application of gaseous nitric oxide as a topical antimicrobial agent.* Nitric Oxide-Biology and Chemistry, 2006. **14**(1): p. 21-29.

122. Hetrick, E.M., et al., *Bactericidal efficacy of nitric oxide-releasing silica nanoparticles.* Acs Nano, 2008. **2**(2): p. 235-246.

123. Ghaffari, A., et al., *Efficacy of gaseous nitric oxide in the treatment of skin and soft tissue infections.* Wound Repair and Regeneration, 2007. **15**(3): p. 368-377.

124. Li, Y. and P.I. Lee, *Controlled Nitric Oxide Delivery Platform Based on S-Nitrosothiol Conjugated Interpolymer Complexes for Diabetic Wound Healing.* Molecular Pharmaceutics, 2009. **7**(1): p. 254-266.

125. Köritzer, J., et al., *Restoration of Sensitivity in Chemo — Resistant Glioma Cells by Cold Atmospheric Plasma.* PLoS ONE, 2013. **8**(5): p. e64498.

126. Volotskova, O., et al., *Targeting the cancer cell cycle by cold atmospheric plasma.* Sci Rep, 2012. **2**: p. 636.

127. Wang, M., et al., *Cold Atmospheric Plasma for Selectively Ablating Metastatic Breast Cancer Cells.* PLoS ONE, 2013. **8**(9): p. e73741.

128. Arndt, S., et al., *Cold Atmospheric Plasma (CAP) Changes Gene Expression of Key Molecules of the Wound Healing Machinery and Improves Wound Healing In Vitro and In Vivo.* PLoS One, 2013. **8**(11): p. e79325.

129. Arndt, S., et al., *Cold atmospheric plasma, a new strategy to induce senescence in melanoma cells.* Exp Dermatol, 2013. **22**(4): p. 284-9.

130. Daeschlein, G., et al., *Cold plasma is well-tolerated and does not disturb skin barrier or reduce skin moisture.* Journal Der Deutschen Dermatologischen Gesellschaft, 2012. **10**(7): p. 509-515.

131. Li, Y.F., et al., *In vivo skin treatment using two portable plasma devices: Comparison of a direct and an indirect cold atmospheric plasma treatment.* Clinical Plasma Medicine, 2013. **1**(2): p. 35-39.

132. Bender, C., et al., *The Irritation Potential of Nonthermal Atmospheric Pressure Plasma in the HET-CAM.* Plasma Processes and Polymers, 2010. **7**(3-4): p. 318-326.

133. Kalghatgi, S., et al., *Mechanism of blood coagulation by non-thermal atmospheric pressure dielectric barrier discharge.* 2007 Ieee Pulsed Power Conference, Vols 1-4, 2007: p. 1058-1063.

134. Cheng, X., et al., *The Effect of Tuning Cold Plasma Composition on Glioblastoma Cell Viability.* PLoS ONE, 2014. **9**(5): p. e98652.

135. Daeschlein, G., et al., *Antibacterial Activity of an Atmospheric Pressure Plasma Jet Against Relevant Wound Pathogens in vitro on a Simulated Wound Environment.* Plasma Processes and Polymers, 2010. **7**(3-4): p. 224-230.

136. Orazov, M., Y. Sakiyama, and D.B. Graves, *Wound healing modeling: investigating ambient gas plasma treatment efficacy.* Journal of Physics D: Applied Physics, 2012. **45**(44): p. 445201.

137. Shekhter, A.B., et al., *Experimental and clinical validation of plasmadynamic therapy of wounds with nitric oxide.* Bulletin of Experimental Biology and Medicine, 1998. **126**(8): p. 829-834.

138. Yawn, B.P. and D. Gilden, *The global epidemiology of herpes zoster.* Neurology, 2013. **81**(10): p. 928-930.

139. Stankus, S.J., M. Dlugopolski, and D. Packer, *Management of herpes zoster (shingles) and postherpetic neuralgia.* Am Fam Physician, 2000. **61**(8): p. 2437-44, 2447-8.

140. Isbary, G., et al., *Randomized placebo-controlled clinical trial showed cold atmospheric argon plasma relieved acute pain and accelerated healing in herpes zoster.* Clinical Plasma Medicine, 2014(0).

141. Isbary, G., et al., *Cold atmospheric plasma: a successful treatment of lesions in Hailey-Hailey disease.* Archives of Dermatology, 2011. **147**(4): p. 388-90.

142. Isbary, G., et al., *Cold atmospheric plasma for local infection control and subsequent pain reduction in a patient with chronic post-operative ear infection.* New Microbes and New Infections, 2013. **1**(3): p. 41-43.

143. Koban, I., et al., *Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro - proof of principle experiment.* Journal of Clinical Periodontology, 2011. **38**(10): p. 956-965.

144. Vandamme, M., et al., *ROS implication in a new antitumor strategy based on non-thermal plasma.* Int J Cancer, 2012. **130**(9): p. 2185-94.

145. Guerrero-Preston, R., et al., *Cold atmospheric plasma treatment selectively targets head and neck squamous cell carcinoma cells.* International journal of molecular medicine, 2014. **34**(4): p. 941-946.

9. Publications

I.

Investigation of the mutagenic potential of cold atmospheric plasma at bactericidal dosages
Boxhammer, V., Li, Y. F., Koritzer, J., Shimizu, T., Maisch, T., Thomas, H. M., Schlegel, J., Morfill, G. E., Zimmermann, J. L.
Mutation research (2013)

II.

Bactericidal action of cold atmospheric plasma in solution
V Boxhammer, G E Morfill, J R Jokipii, T Shimizu, T Klämpfl, Y-F Li, J Köritzer, J Schlegel, J L Zimmermann
New Journal of Physics (2012)

III.

In vivo skin treatment using two portable plasma devices: Comparison of a direct and an indirect cold atmospheric plasma treatment
Li, Y. F., Taylor, D., Zimmermann, J. L., Bunk, W., Monetti, R., Isbary, G., Boxhammer, V., Schmidt, H. U., Shimizu, T., Thomas, H. M., Morfill, G. E.
Clinical Plasma Medicine (2013)

IV.

Disinfection Through Different Textiles Using Low-Temperature Atmospheric Pressure Plasma
Zimmermann, J. L., Shimizu, T., Boxhammer, V., Morfill, G. E.
Plasma Processes and Polymers (2012)

V.

Effects of cold atmospheric plasmas on adenoviruses in solution
Zimmermann, J L, Dumler, K, Shimizu, T, Morfill, G E, Wolf, A, Boxhammer, V, Schlegel, J, Gansbacher, B, Anton, M
Journal of Physics D: Applied Physics (2011)

Publication, which is not part of this thesis:

Restoration of Sensitivity in Chemo —
Resistant Glioma Cells by Cold Atmospheric Plasma
Köritzer, Julia, Boxhammer, Veronika, Schäfer, Andrea, Shimizu, Tetsuji, Klämpfl, Tobias G., Li, Yang-Fang, Welz, Christian, Schwenk-Zieger, Sabina, Morfill, Gregor E., Zimmermann, Julia L., Schlegel, Jürgen
PLoS ONE (2013)