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Efficacy of Different Carrier Gases for Barrier Discharge Plasma Generation Compared to Chlorhexidine on the Survival of *Pseudomonas aeruginosa* Embedded in Biofilm in vitro

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Key Words

Biofilm · Wound management · Antimicrobial · *Pseudomonas aeruginosa* · Atmospheric pressure plasma · Surface barrier discharge plasma source · Dielectric barrier discharge

Abstract

Because of its antimicrobial properties, nonthermal plasma could serve as an alternative to chemical antisepsis in wound treatment. Therefore, this study investigated the inactivation of biofilm-embedded *Pseudomonas aeruginosa* SG81 by a surface barrier-discharged (SBD) plasma for 30, 60, 150 and 300 s. In order to optimize the efficacy of the plasma, different carrier gases (argon, argon admixed with 1% oxygen, and argon with increased humidity up to approx. 80%) were tested and compared against 0.1% chlorhexidine digluconate (CHG) exposure for 600 s. The antimicrobial efficacy was determined by calculating the difference between the numbers of colony-forming units (CFU) of treated and untreated biofilms. Living bacteria were distinguished from dead by fluorescent staining and confocal laser scanning microscopy. Both SBD plasmas and CHG showed significant antimicrobial effects compared to the untreated control. However, plasma treatment led to a higher antimicrobial reduction (argon plasma 4.9 $\log_{10}$ CFU/cm^2, argon with admixed oxygen 3 $\log_{10}$ CFU/cm^2, and with increased gas humidity 2.7 $\log_{10}$ CFU/cm^2 after 300 s) compared to CHG. In conclusion, SBD plasma is suitable as an alternative to CHG for inactivation of *Pseudomonas aeruginosa* embedded in biofilm. Further development of SBD plasma sources and research on the role of carrier gases and humidity may allow their clinical application for wound management in the future.

Introduction

Bacterial biofilms are described as attached bacteria on surfaces embedded in a matrix composed of self-produced substances, mainly polysaccharides, organic material from the immediate environment and water [1, 2].

Bacterial biofilm has been shown to play a role in delayed wound healing [3–6]. *Pseudomonas aeruginosa* is one of the most important biofilm-forming species in chronic wounds [7]. This organism, however, is difficult to eradicate as it is protected against antimicrobial compounds by its biofilm, while at the same time it produces a number of enzymes, e.g. matrix metalloproteinases or toxins such as exotoxin A, which inhibit wound healing [8, 9].

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While mechanical debridement is the most effective measure for removing biofilm, topical antimicrobial compounds are also used to control and eradicate microorganisms inhabiting biofilms [10, 11]. Combining physical and antimicrobial measures to remove biofilm and to kill off embedded microorganisms would be of added value in wound management [12, 13].

The antimicrobial effect of plasma is mainly based on its reactive oxygen species (ROS), reactive nitrogen species and UV radiation [14–16]. While the basic principles of biological plasma effects have been studied extensively in recent years, data on optimal parameters for independent variables (e.g. type of source, exposure time, gas mixture) differ between different application forms. However, determining the optimal adjustment of parameters from the experimental setups is crucial for developing clinical applications for future clinical use.

The present study investigated the antimicrobial efficacy of a nonthermal atmospheric pressure argon (Ar) plasma generated by a surface barrier-discharged (SBD) plasma source with different modifications (exposure time, gas mixtures) on in vitro biofilms of *P. aeruginosa* in comparison to chlorhexidine digluconate (CHG). CHG is used in dentistry and represents the gold standard to inhibit plaque formation [17]; it is also used on wounds for wound antisepsis [18–20]. Hence, CHG was chosen to serve as the positive control and additionally as a control of biofilm stability (susceptibility to antimicrobial treatment).

Because the plasma source used in this study also emits UV light, a risk assessment of UV irradiation on skin was conducted by measuring UV emission.

Materials and Methods

Characteristics of the Plasma Source

The SBD plasma source (Neoplas GmbH, Greifswald, Germany) was developed by the Leibniz Institute for Plasma Science and Technology (INP), Greifswald, Germany. The plasma source works with sinus 30 kHz at U_{pp} = 4 kV in a burst mode (250 ms on-time, 750 ms off-time) and covers an active discharge surface of 11 cm^2. The electrical power was adjusted to 1.3 W, determined using a Lissajous curve (error limits: 10–20%) [21, 22], resulting in a surface power of 118 mW/cm^2. A metal electrode was grounded

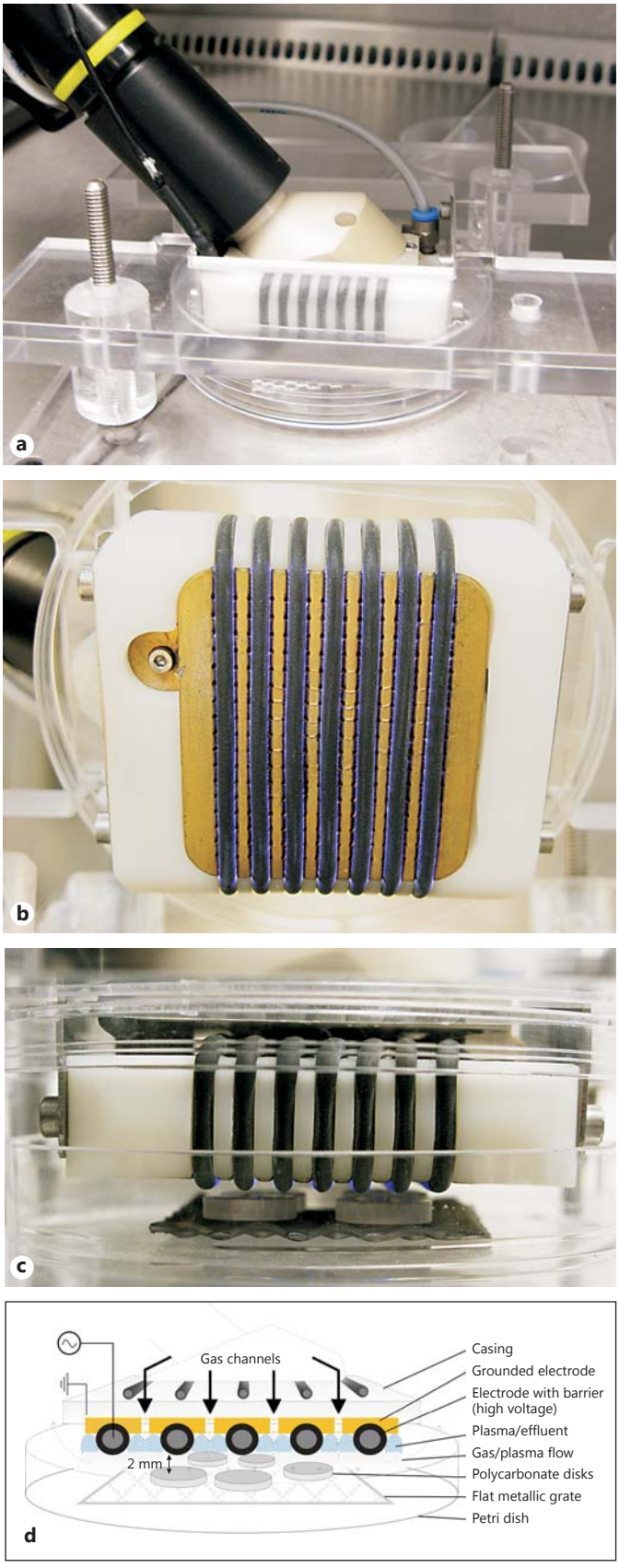

Fig. 1. Experimental setup of the SBD plasma source. **a** SBD plasma source fixed in casing for action. **b** Bottom of the SBD plasma source in action. **c** Plasma treatment of biofilm-covered disks. **d** Schematic depiction of SBD plasma source (cross-section).

for safety and connected to 7 black silicone-encased wires (thickness: 0.6 mm each) serving as the dielectric barrier electrode. Construction and experimental setup of the SBD plasma source are depicted in figure 1.

The construction of this SBD electrode is applicable to different sizes and shapes; hence, the surfaces can be custom treated as the application demands, making it highly suitable for real-life applications [23]. Additionally, the SBD electrode avoids direct discharges to the substrate while always operating at room temperature near the electrodes [24]. These physical properties make this SBD suitable for use as a tissue-tolerable plasma source.

UV Radiation Measurement of the Plasmas

For all application modes, the exposure to UV radiation in the range of 180–400 nm was measured in microwatts per square centimeter at a constant distance of 2 mm using spectrometry (Avantes AvaSpec-3648, Apeldoorn, The Netherlands). The area under the curve of the graph was calculated. The UV radiation exposure was determined in millijoules per square centimeter in accordance with ICNIRP guidelines (guidelines on limits of exposure to UV radiation of wavelengths between 180 and 400 nm) [25]. The erythema risk was determined in accordance with the Scientific Committee on Consumer Products report [26].

Cultivation and Evaluation of Experimental Biofilms

For the preparation and formation of a reproducible biofilm, the strongly biofilm-forming *P. aeruginosa* strain SG81 was used, as described earlier [19, 27, 28]. The biofilms were cultivated on polycarbonate disks (diameter 13 mm, height 3 mm; Arthur Krueger KG, Barsbüttel, Germany) submerged in an artificial wound-like fluid consisting of minimal essential medium and 10% fetal bovine serum (Gibco-Invitrogen, Germany) [29] serving as growth medium. The batch medium had a final concentration of 10^8 colony-forming units (CFU)/ml. Sterile disks were positioned in 24-well microplates (Sarstedt AG & Co., Nümbrecht, Germany), covered with 0.8 ml batch medium, and incubated aerobically for 72 h in an agitator system (Polymax, Heidolph, Germany) at 150 revolutions per minute (r.p.m.). Medium was replaced after 24 and 48 h with sterile artificial wound medium, and the polycarbonate disks were turned inside the respective wells. The incubation temperature was set to 37°C for the first 48 h, and 23°C for the following 24 h.

The final disks covered with biofilm were washed with phosphate-buffered saline solution to remove unattached bacteria and were transferred to sterile microplates for the subsequent experiments.

Finally, after the experiments, all disks were placed in microplates, were filled with 1 ml of physiological saline solution per well, and treated by ultrasound (130 W, Branson 2510 Ultrasonic Cleaner, Emerson Technologies GmbH & Co. OHG, Dietzenbach, Germany) for 20 min to disperse the biofilms. Planktonic bacteria were determined by the spread plate method on tryptic soy agar (CASO-Agar, Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

Plasma Experiments on Biofilms

Biofilm-covered carrier disks were positioned on a flat metallic grate, in order to keep the contact area at a minimum, and placed in a petri dish. The SBD plasma source was positioned above the disks and immobilized by fixing at 3 points (fig. 1). In each experiment, the distance between the dielectric wire and the surface of the disks was adjusted to 2 mm with the support of the casing of the plasma source. A gas flow of 5 standard liters/min was applied through small holes along the black wires in the metal electrode and was controlled by a mass flow controller (MKS Instruments, Munich, Germany). Ambient air exchange was allowed through the gap between the electrode and the edge of the petri dish.

Plasma or gas control was applied using Ar (99.995% pure), Ar admixed with 1 vol% oxygen (Ar + O_2, O_2 99.5% pure), or Ar with 80% relative gas humidity (Ar + H_2O). The water content of the pure Ar and O_2 gas was <3 ppm/mol. In all assays, both sides of the disks were treated for 30, 60, 150 or 300 s. In order to increase the relative humidity to 80 ± 4% (at 21°C) for Ar, the gas flow was piped through a bottle containing distilled water. The resulting humidity was measured using a hygrometer (testo 400, Lenzkirch, Germany).

Antimicrobial Treatment of Biofilms Using CHG as Antiseptic Reference

For treatment with CHG instead of plasma, biofilm-covered carrier disks were also placed in microplate wells, filled with 0.9 ml of a 0.1% (w/v) CHG solution (Fagron GmbH & Co. KG, Barsbüttel, Germany) and incubated for 600 s. Antiseptic activity was neutralized by replacing CHG with 1 ml of inactivation solution for 600 s (40 g/l Tween 80, 30 g/l saponin, 4 g/l lecithin, 10 g/l sodium dodecyl sulfate, 1 g/l sodium thioglycolate; Serva, Heidelberg, Germany). The efficacy of the neutralizer was validated following the Standard European Norm DIN EN 1040 [30].

Staining of Biofilm and Microscopic Evaluation

Two fluorescent nucleic acid stains, Syto® 9 (Syto9) and propidium iodide (Live/Dead-BacLight, Invitrogen GmbH, Darmstadt, Germany), were used for discrimination of intact (stained with Syto9, green signals) and membrane-damaged (stained with propidium iodide, red signals) bacteria [31]. The treated experimental and untreated control biofilm disks were transferred into 24-well microplates (Techno Plastic Products AG, Trasadingen, Switzerland) and covered with 0.5 ml of staining solution (6.7 μM of Syto9 and 5 μM of propidium iodide) for 30 min at 80 rpm on an agitator system (IKA® KS 130 basic, Staufen, Germany) in the dark. The staining solution was removed, and the disks were washed with 1 ml PBS. Finally, the stained biofilm disks were transferred into a new 24-well microplate. A confocal laser scanning microscope (Zeiss CLSM510 Exciter, Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with a ×10 objective (Zeiss EC Plan-Neofluar ×10/0.3) was used for imaging.

Statistical Analysis

CFU counts per square centimeter were transformed to $\log_{10}$ (CFU) per square centimeter. For each sample the colony reduction factor (RF) was calculated by subtracting the $\log_{10}$ (CFU) per square centimeter value of the treated sample from the $\log_{10}$ (CFU) per square centimeter of the mean value of the untreated control. For each condition, means and standard deviations were calculated based on the RFs. Statistical differences between RFs of different treatment modalities and times were analyzed with the Kruskal-Wallis test, followed by a Mann-Whitney U test using the statistical analysis software SAS® Enterprise Guide® 4.1 (SAS Institute GmbH, Heidelberg, Germany).

Table 1. Summary of results

Treatment mode	Ar				$Ar + O_2$				$Ar + H_2O$				p
	n	RF ± SD	95% CI limits		n	RF ± SD	95% CI limits		n	RF ± SD	95% CI limits		
			lower	upper			lower	upper			lower	upper	
Plasma													
30 s	22	1.15±0.99[a]	0.72	1.59	14	1.66±1.20[c]	0.97	2.36	6	0.97±0.37	0.58	1.36	0.2242
60 s	21	1.63±0.67[b, c]	1.32	1.94	13	2.19±1.28[c]	1.42	2.97	12	1.19±0.86	0.64	1.73	0.0438
150 s	21	2.45±1.03[a, b]	1.98	2.91	14	2.62±0.95[a, b]	2.07	3.17	6	1.55±0.93	0.58	2.52	0.0976
300 s	14	4.88±2.21[a–c]	3.60	6.15	12	2.97±1.64[a]	1.93	4.01	6	2.65±1.21[a, b]	1.38	3.92	0.0666
p		0.0001				0.0355				0.0313			0.0001
Gas													
30 s	6	0.66±0.50[a]	0.13	1.18	7	1.94±1.19	0.84	3.05	6	0.85±0.65	0.17	1.53	0.0784
60 s	6	0.62±0.26[a]	0.35	0.88	7	1.41±0.53	0.97	1.85	6	0.56±0.56	–0.03	1.14	0.0135
150 s	14	1.11±0.83	0.63	1.59	12	0.79±0.43	0.52	1.07	6	1.28±1.10	0.12	2.43	0.6751
300 s	11	1.52±0.80	0.98	2.05	8	1.97±1.31	0.87	3.06	6	0.47±0.30	0.16	0.79	0.0052
p		0.0153				0.018				0.3589			0.4598
CHG 0.1%	22	1.41±0.73	1.08	1.73									
Control	36	0.00±0.340	–0.19	0.06									
p		0.0001				0.0001				0.0001			0.0001

Number of samples (n), RF expressed as $\log_{10}$ CFU per square centimeter ± standard deviation (SD), lower and upper 95% confidence intervals (CI) after Ar plasma, $Ar + O_2$ plasma and $Ar + H_2O$ plasma treatment for 30–300 s treatment time and for 300 s treatment time of respective gas controls, 0.1% CHG after 600 s exposure time and untreated control of *P. aeruginosa* SG81 biofilms; p values of omnibus tests (Kruskal-Wallis) and two-sample tests (Whitney U); statistical significance: $\alpha = 0.05$. [a] $p \leq 0.05$: significantly different from CHG; [b] $p \leq 0.05$: significantly different from the respective gas control; [c] $p \leq 0.05$: significantly different from the respective $Ar + H_2O$ plasma treatment time.

Results

Regardless of the respective tested condition, all plasma treatments resulted in a measurable microbial reduction ranging between 0.97 $\log_{10}$ CFU/cm^2 ($Ar + H_2O$ plasma) and 4.88 $\log_{10}$ CFU/cm^2 (Ar plasma), compared to untreated controls ($p < 0.009$). The RFs increased with longer and decreased with shorter treatment time, showing a strong correlation with the plasma exposure time.

The antimicrobial efficacy of CHG (1.4 $\log_{10}$ CFU/cm^2) after 600 s was reached by Ar plasma within 60 s and by $Ar + O_2$ plasma within 30 s. $Ar + H_2O$ plasma was less effective, reaching the antimicrobial reduction efficacy of CHG after 150 s. For longer treatment times, reductions were significantly superior ($p < 0.014$) to CHG treatment (table 1).

Plasma control with pure Ar gas flow also resulted in significant bacterial reduction (maximum reduction was 1.5 $\log_{10}$ for Ar gas, 2 $\log_{10}$ for $Ar + O_2$ gas and 0.9–1.3 $\log_{10}$ for $Ar + H_2O$ gas) compared to the untreated control, yet the RFs were smaller and showed no correlating time dependency. The antimicrobial efficacy shown by $Ar + H_2O$ gas control was always low, with an RF in the range of approximately 1 $\log_{10}$ CFU/cm^2. The difference from the negative control was not statistically significant ($p = 0.36$). However, plasma was significantly more effective for the carrier gas Ar after 60–300 s and for $Ar + H_2O$ after 300 s of exposure compared to the gas treatment

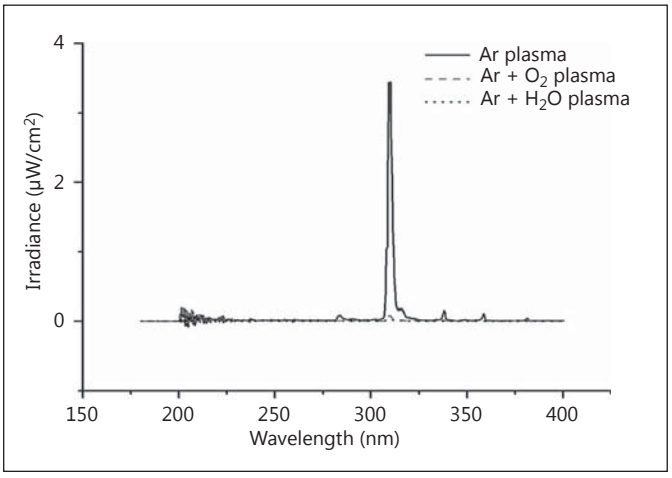

Fig. 2. Spectrometric graph of irradiance by generated Ar, Ar with 1% O_2 admixture and Ar with 80% gas humidity plasma during 300 s of exposure time between 180 and 400 nm.

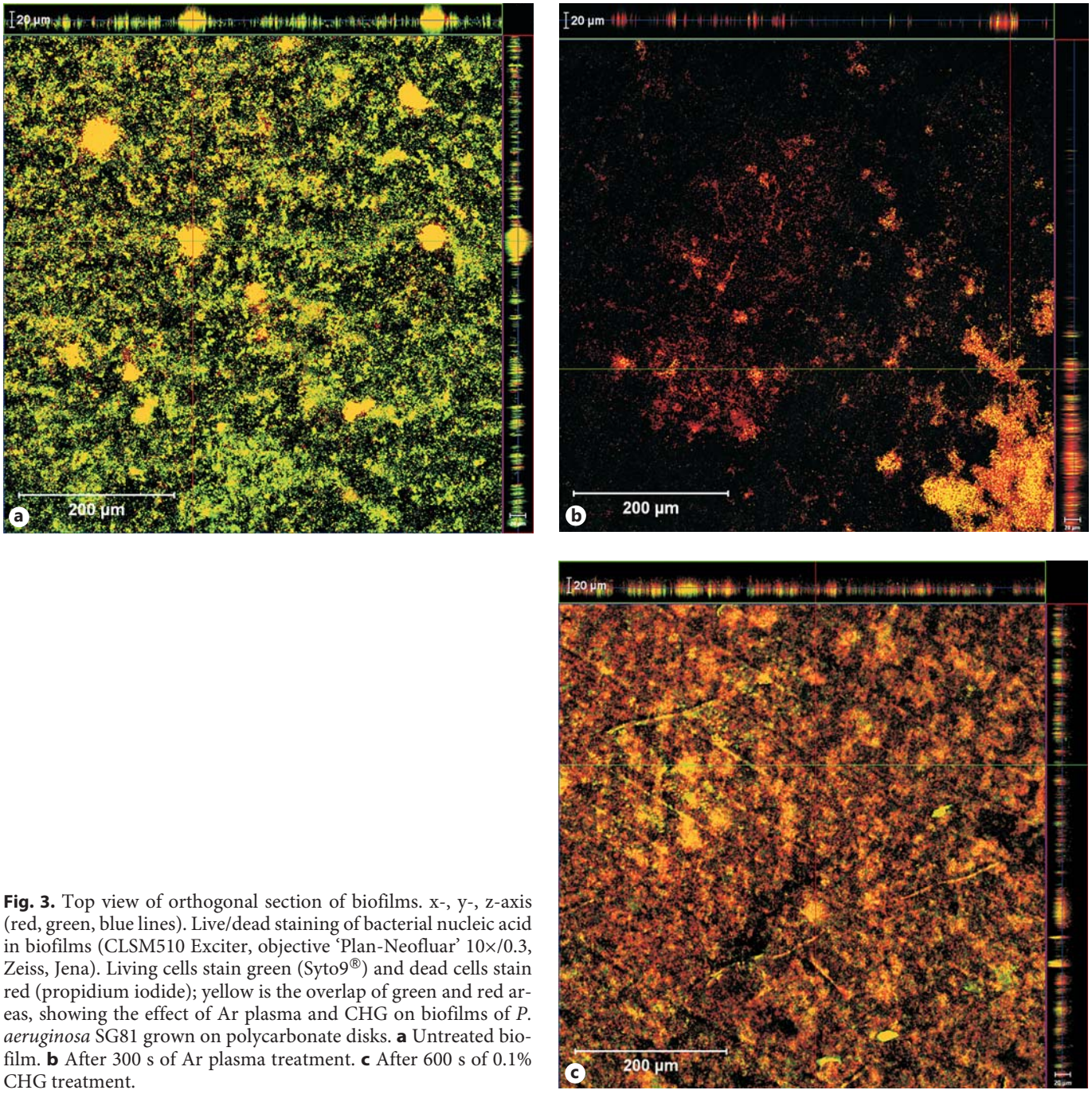

Fig. 3. Top view of orthogonal section of biofilms. x-, y-, z-axis (red, green, blue lines). Live/dead staining of bacterial nucleic acid in biofilms (CLSM510 Exciter, objective 'Plan-Neofluar' 10×/0.3, Zeiss, Jena). Living cells stain green (Syto9®) and dead cells stain red (propidium iodide); yellow is the overlap of green and red areas, showing the effect of Ar plasma and CHG on biofilms of *P. aeruginosa* SG81 grown on polycarbonate disks. **a** Untreated biofilm. **b** After 300 s of Ar plasma treatment. **c** After 600 s of 0.1% CHG treatment.

alone ($p < 0.004$). The efficacy of Ar + O_2 plasma after 60 s of exposure was higher than the respective gas treatment serving as control. However, the differences of the RFs were not statistically significant.

Increasing the relative humidity of the carrier gas did not lead to greater antimicrobial efficacy; on the contrary, the RF of Ar + H_2O plasma was significantly lower than that of Ar plasma after 300 s ($p = 0.0408$).

The maximum UV irradiation from the plasma source was 14.6 µW/cm^2, which was measured during the use of pure Ar plasma. This UV value corresponded to a UV radiation exposure of 0.32 mJ/cm^2 during 300 s of treat-

ment. The other gas variations showed lower UV radiation exposures of 0.07 mJ/cm^2 by Ar + O_2 plasma, and 0.03 mJ/cm^2 by Ar + H_2O plasma during 300 s of treatment. The highest measured erythema dose was 0.0032 W/m^2 for Ar plasma. A spectrometric graph of the UV irradiance by Ar, Ar + O_2 and Ar + H_2O plasma is shown in figure 2.

The biofilm thickness of 24.5–30.6 μm remained unchanged after treatment for nondetached, stainable biofilms. Figure 3 shows the fluorescence images of plasma- and CHG-treated (positive control) as well as untreated negative control biofilms. Image analysis of biofilms after plasma treatment showed a smaller area of fluorescence with irregularly distributed red signals in comparison to the other samples. After CHG treatment, the distribution of red signals was notably superficial as opposed to deeper, greener signals before CHG treatment.

Discussion

Treatment of bacterial biofilms by application of physical plasma is of special interest, because this could be an alternative method to the application of chemical antimicrobials. It offers new options for inactivating microorganisms inhabiting biofilms on prosthetic material or chronic surface tissue infections, e.g. in chronic wounds.

To ascertain the safety of the SBD-generated plasma for possible applications on human skin, we quantified the UV emission. Other than solid inanimate surfaces, human cells in wounds may be damaged by UV irradiation, particularly in the wavelength range of 180–400 nm [25, 32–34]. At high doses, UV radiation may not only cause cell damage and lead to apoptosis but may also induce cancer [35]. Depending on the gas or gas mixture used, the measurements showed that the UV radiation emitted by the SBD plasmas ranged between 0.03 mJ/cm^2 (Ar + H_2O plasma) and 0.32 mJ/cm^2 (1.06 μW/cm^2; Ar plasma) for 300 s of application time at a distance of 2 mm (comparable to distance during biofilm treatment). In humans, the maximum accepted UV radiant exposure limit for intact skin over 300 s is 3 mJ/cm^2 (10 μW/cm^2), as stated in the ICNIRP guidelines [25]. Additionally, the weighted total erythema irradiance of 0.0032 W/cm^2 is clearly below the erythema risk threshold (0.3 W/m^2), in accordance with the Scientific Committee on Consumer Products report [26]. The UV and erythema irradiance determined was lower than the reported UV exposure produced by the Ar plasma source which was used to treat chronic wounds [36]. These results indicate that the UV irradiation of the SBD plasma is negligibly low, which is one important prerequisite for the use of plasma in human wounds.

The effect of various gas admixtures for optimized antimicrobial efficacy in biofilms was investigated in this study. Up to now, no accepted standard for testing antimicrobial compounds against microbial biofilms has been defined. Therefore, the described assay was developed, which proved reliable and easy to use for the research questions posed here. The untreated controls showed means of 7.14 ± 0.8 $\log_{10}$ CFU/cm^2 and were suitable for calculating the RFs for statistical analyses (in accordance with DIN EN 1040 [30]). These high colony counts were achievable by using a distinct *P. aeruginosa* test strain [37]. Using this strain, pretests showed a stronger biofilm structure if the incubation temperature was decreased to room conditions for the last 24 h of incubation while more intense bacterial growth occurred during the first 48 h at 37°C. An artificial wound fluid was used to simulate a wound-like environment during biofilm culture. Polycarbonate disks were used as the adhesion surface. This material is suitable for the adherence and biofilm formation of *P. aeruginosa* [38].

Chlorhexidine is used in topical antimicrobial wound dressings and is also regarded as the gold standard to eradicate dental plaque and consequently inactivate and prevent biofilms [39]. Therefore, CHG was used in the present experiments as the positive control. It demonstrated a significant bacterial reduction of 1.4 $\log_{10}$ CFU/cm^2 after 600 s of application ($p < 0.0001$). It was expected that any plasma treatment must at least demonstrate antimicrobial reduction efficacy equal to or significantly higher than CHG compared to the untreated negative control.

In our experiments, the efficacy of Ar plasma and of Ar + O_2 plasma after 150 s and of Ar + H_2O plasma after 300 s was higher than the antimicrobial efficacy of CHG. The RFs achieved by the various plasma conditions are in agreement with similar previously published studies [19, 40].

Ar showed a strong antimicrobial effect, which increased steadily with increasing plasma exposure time from approximately 1 to 4.9 $\log_{10}$ (CFU)/cm^2. This reduction rate is comparable to the antimicrobial efficacy of a helium [41] or an Ar [42] plasma jet against biofilms of *P. aeruginosa*. Yet not only Ar, but also the Ar gas flow showed an antimicrobial effect, which was also reported elsewhere for an Ar plasma jet on biofilms [42]. This observation may be explained by a simple drying effect of the biofilm by the gas flow itself, since the effect was sig-

nificantly reduced when humidity was added in the experiments using Ar + H_2O gas. However, the Ar plasma effect was significantly higher than the pure gas effect alone for 60–300 s of exposure time.

The SBD plasma source used for this study allowed admixing O_2 to the Ar gas or enriching it with distilled water. By adding O_2 or increasing humidity, an increased concentration of ROS (e.g. O, O_2^-, OH, H_2O_2, $^1O_2$, O_3) was expected [43, 44]. Especially the electrons of Ar plasma have enough energy to dissociate O–H bonds of water, so the gas humidity may increase the concentration of OH radicals [45]. ROS are essentially downstream products of the plasma due to its reaction with room air or with water on the substrate and result in increased antimicrobial efficacy [45, 46].

Surprisingly, the increased humidity decreased the plasma's antimicrobial efficacy against *P. aeruginosa* in treated biofilms. Other authors reported an increased antimicrobial effect with additional gas humidification [47–49]. Hähnel et al. [47] observed an improved effect for an air plasma by increasing the relative air humidity up to 70% against *Bacillus atrophaeus* spores. The authors discussed that especially hydroxyl radicals have an erosive effect on cell surfaces. Indeed, it is known that a very small amount of moisture on the microbial target is needed for inactivation with species such as ·OH or H_2O_2 [48–50]. This could explain why an increased effect was observed when humidified gas was applied on dry spores. While in the experiments on *B. atrophaeus* spores [47] only the amount of water needed for the plasma decontamination process was used, the biofilm in our experiments already contained the water necessary for antimicrobial effects, leaving no chance for enhancement due to humidification. Dobrynin et al. [48] observed an enhanced antimicrobial efficacy by carrier gas humidification (bubbled through distilled water) only if additional O_2 was admixed. Here, the relative gas humidity could be the same as in the present study and our plasma source worked in ambient air, which could provide the additional O_2. Srivastava and Wang [49] observed an enhanced effect with increased humidity alone in Ar plasma, yet with a lower relative water content than used in our study. According to those authors, an H_2O:Ar ratio of 1.5% was optimal for acquiring high amounts of OH radicals in plasma effluvium. The increase in antimicrobial effectiveness seems to be very small, because increasing the relative humidity to 20–80% did not significantly change the efficacy of air plasma [51]. Consequently, the relative humidity of 80% in Ar could have been set too high.

All groups of authors used different plasma devices, carrier gases, or application systems, but none focussed on the determination of the plasma effect against biofilm bacteria or worked in ambient conditions in the way described in the present study, making direct comparison difficult. Finally, the high gas humidity of 80% in Ar could have led to a loss of electron density for plasma generation [45] that may have inhibited important antimicrobial components, explaining why the antimicrobial efficacy of Ar + H_2O plasma was lower than that of Ar plasma. Therefore, a higher energy input for plasma generation or lower Ar humidity may be able to increase the efficacy of Ar + H_2O plasma. Another explanation could be a shift of different ROS generated to H_2O_2 by the added H_2O vapor [52], since *P. aeruginosa* can neutralize these peroxides by producing catalases [53], showing increased resistance against H_2O_2 in biofilms [54]. Further investigations are necessary to understand this phenomenon, because the plasma chemistry of humidified air plasma is very different from humidified Ar plasma, and from humidified Ar plasma working in ambient conditions. The ambient conditions for plasma treatment were chosen to simulate the clinical conditions of wound treatment on patients.

Correlations between the antimicrobial effects of plasma and biofilm water content should be a topic of future studies.

The results of this study demonstrated that the addition of 1% O_2 to Ar accelerated the onset of the antimicrobial effect, but with longer treatment time, the efficacy decreased again, particularly in comparison to Ar plasma alone. Comparable results were shown by the use of an Ar plasma jet [42]. Possibly, the admixture of 1% O_2 quenches the excited Ar species [55] and inhibits secondary plasma products which are important for potential antimicrobial effects against *P. aeruginosa*. Otherwise, on scanning electron images, some other authors have shown cell detritus after plasma treatment [40, 56, 57]. It may well be that an increased O_2 concentration accelerates microbial cell damage, which may result in a plasma shadow effect by accumulated cell detritus. This could explain why the antimicrobial efficacy of Ar + O_2 plasma increased very slowly with longer application times.

In order to take advantage of possible ROS by O_2 admixtures for treatment of *P. aeruginosa* in biofilms, further studies on optimizing the Ar:O_2 ratio in plasma are required.

The antimicrobial effect after treatment with Ar + O_2 gas was higher than after Ar gas treatment and cannot be explained only by the drying effect of the biofilm. It is

known that relatively low O_2 concentrations dominate within *P. aeruginosa* biofilms (vs. on their surface) [58, 59] and that pure O_2 can diffuse more easily through the biofilm matrix than can air, for example [58]. The initially O_2-poor status of biofilm to which O_2 was added could explain the effect of the rapid onset within 30 s, the slow progress with longer exposure to Ar + O_2 plasma, and the high antimicrobial effect of Ar + O_2 gas flow with increased O_2 stress of *P. aeruginosa*. The difference between Ar + O_2 gas and Ar + O_2 plasma is only 1 $\log_{10}$ after 300 s of exposure and was not significant. In comparison to the other treatment times, the low effect of 0.8 $\log_{10}$ CFU/cm^2 at 150 s by Ar + O_2 gas is currently inexplicable, because the procedure was constant for all samples.

To avoid a dominant gas flow effect for efficient antimicrobial reduction of microorganisms in biofilms, treatment with Ar or Ar + H_2O plasma is recommended.

The fluorescence images showed darker regions after plasma treatment. This could be a result of reduced adherence of biofilm, or reduced staining may have been due to loss of DNA caused by the plasma effect and the additional washing procedure. Furthermore, the distribution of live and dead *P. aeruginosa* cells was different between plasma- and CHG-treated biofilms. The z-axis of the images after CHG treatment shows more red signals (dead bacteria) in the surface regions of the biofilm structure and green signals (live bacteria) in deeper regions, while signals were distributed irregularly after 300 s of Ar plasma treatment. This indicates that the antimicrobial efficacy of Ar plasma is homogeneously distributed through the biofilm structure, while CHG did not reach deeper layers of the biofilm. A homogeneously distributed effect of plasma in deeper regions of the biofilms was also shown by other authors [13, 41, 60].

While the assay allowed investigation of plasma effects under standardized conditions, this experimental biofilm does not correspond to complex multispecies biofilms found in vivo as in chronic infected wounds [61]. Additionally, the experimental model used in this study does not correspond to the complex wound conditions found in chronic wounds. Finally, such well-defined physical conditions during plasma treatment as were present in our experimental tests are not found in the clinical setting, since differences in the distance of the plasma source to the surface, in room temperature, and in humidity conditions are to be expected.

Conclusion

The results of this study demonstrate that the high antimicrobial efficacy of the SBD plasma source used against *P. aeruginosa* embedded in biofilm exceeds that of CHG applied for 600 s. SBD plasma may be suitable as an alternative or supplemental treatment to chemical antiseptics in the treatment of biofilm-associated wound infections. These effects, however, depend on chemical and physical factors, such as the plasma gas condition, but also the physical condition of the surrounding air. The admixture of O_2 to Ar for plasma generation led to an increased antimicrobial effect, albeit only for short treatment times. Concurrently, an increased humidity of Ar of approximately 80% to Ar for plasma generation reduced the plasma's antimicrobial effect in the presence of biofilm. Further basic research on SBD plasma sources and on the role of carrier gases and humidity is needed to better understand the complex plasma physics in clinical practice.

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Disclosure Statement

Klaus-Dieter Weltmann is a minority shareholder of Neoplas Tools GmbH, Greifswald, Germany, and member of the consulting committee of Optical Technologies (BMBF). The INP Greifswald is a majority shareholder of Neoplas GmbH.

All other authors declare no conflicts of interest.

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