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Abstract. Because of some disadvantages of chemical disinfection in dental practice (especially denture cleaning), we investigated the effects of physical methods on *Candida albicans* biofilms. For this purpose, the antifungal efficacy of three different low-temperature plasma devices (an atmospheric pressure plasma jet and two different dielectric barrier discharges (DBDs)) on *Candida albicans* biofilms grown on titanium discs *in vitro* was investigated. As positive treatment controls, we used 0.1% chlorhexidine digluconate (CHX) and 0.6% sodium hypochlorite (NaOCl). The corresponding gas streams without plasma ignition served as negative treatment controls. The efficacy of the plasma treatment was determined evaluating the number of colony-forming units (CFU) recovered from titanium discs. The plasma treatment reduced the CFU significantly compared to chemical disinfectants. While 10 min CHX or NaOCl exposure led to a CFU $\log_{10}$ reduction factor of 1.5, the $\log_{10}$ reduction factor of

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DBD plasma was up to 5. In conclusion, the use of low-temperature plasma is a promising physical alternative to chemical antiseptics for dental practice.

Contents

1. Introduction

Oral candidosis is an opportunistic infection (caused by pathogens that usually do not cause disease in a healthy host) of the oral cavity with a broad spectrum of clinical signs and symptoms (e.g. denture stomatitis, pseudomembranous candidosis, erythematous candidosis and angular cheilitis) [5]. Oral yeast infections are predominantly caused by *Candida albicans* (*C. albicans*) [6]. In approximately 40% of the total human population and 75% of persons with dentures, the oral cavity is colonized with *C. albicans* forming biofilms.

Biofilm formation on denture surfaces is crucial to the pathogenesis of most oral yeast infections. *C. albicans* is able to colonize and penetrate into pits and fissures of rough denture surfaces. Thus the initial colonization of commensal (part of the human flora) *Candida* could be regarded as the origin of infections. Extracellular polysaccharides (EPS) secreted by *C. albicans* after adhesion on dentures are the basis for the biofilm matrix, which protects the cells from different physical and chemical environmental influences. It has been repeatedly confirmed that *C. albicans* biofilms are more resistant to antifungal agents than planktonic (free-floating cells, non-adherent, without EPS) *C. albicans* cells [13].

Approximately 10–75% of denture wearers present a denture-related stomatitis (inflammation of the mucous membrane of any of the structures in the mouth) caused by *C. albicans* biofilms [2]. Denture stomatitis is an erythematous (redness of the mucous membrane) pathogenic condition of the denture-bearing mucosa [22]. The main reservoir of *C. albicans* is the fitting surface of the upper partial and complete dentures [26]. Denture cleaning should therefore include the removal of *Candida* species. Chemical cleansing baths with peroxides, hypochlorites or chlorhexidine (CHX) digluconates in which dentures are immersed are suggested as the first choice of plaque control. The disadvantages of these agents

comprise a discolouring of dentures by oxygenating agents and black staining in the case of CHX. Also, ultrasonic cleansing baths are not effective [15].

The use of low-temperature plasma as a physical alternative to chemical antiseptics appears to be an interesting alternative due to the dose-dependent microbicidal effectiveness [32]. Furthermore, neither decolouration nor staining of dentures has been observed.

Physical plasma is an electrically neutral, highly ionized gas composed of ions, electrons, excited atoms and molecules, vacuum ultraviolet and ultraviolet (VUV/UV) irradiation, free radicals and chemically reactive neutral particles. There are many possibilities of producing nonthermal plasma at atmospheric pressure [27].

In the present study, we tested the antifungal potential of different plasma devices (atmospheric pressure plasma jet (APPJ); kINPen® 09), hollow electrode dielectric barrier discharge (DBD) and volume DBD to reduce *C. albicans* biofilms *in vitro*.

2. Experimental part

2.1. Biofilm formation

Candida albicans ATCC 10231 (ATCC is American Type Culture Collection, Rockville, MD, USA), a strongly biofilm-forming strain, was used for the tests [14]. Biofilms were cultured on titanium discs with a diameter of 5 mm and a thickness of 1 mm (Straumann, Basel, Switzerland) using standard conditions, as published before [16]. Briefly, *C. albicans* was cultured overnight at 37 °C on Columbia sheep blood agar (BBL™, BD, Heidelberg, Germany). One inoculation loop of this culture was resuspended into 30 ml of YPD broth (Yeast Extract Peptone Dextrose; a complete medium for yeast growth, Sigma, Steinheim, Germany). The sterile test objects were positioned in 96-well microtitre plates (Techno Plastic Products AG, Trasadingen, Switzerland), covered with 100 μl of microorganism suspension and incubated aerobically at 37 °C. Every 24 h the medium was changed. After 48 h the medium was drawn off, and the discs were washed with 0.9% NaCl solution and transferred into a new, sterile microtitre plate.

After plasma treatment, the titanium discs were placed into wells with 200 μl of 0.9% NaCl solution and the biofilm was removed by ultrasonic scaling (20 min). Serial dilutions of this resuspended biofilm solution were made by transferring 0.1 ml of the resultant suspension to 0.9 ml of freshly prepared 0.9% NaCl solution. Afterwards an aliquot portion of 0.1 ml from each dilution was plated on sabouraud glucose (4%) agar plates (Carl Roth, Karlsruhe, Germany) and incubated at 37 °C for 48 h. The colonies were counted and expressed as colony-forming units (CFU). The $\log_{10}$ reduction factor (RF) for each contact time was calculated according to the formula [25]: $\mathrm{RF} = \log_{10} n_\mathrm{c} - \log_{10} n_\mathrm{u}$, where n_c is the number of viable cells (CFU) in the resuspended biofilm solution of NaCl control (in the presence of 0.9% NaCl solution); and n_u is the number of viable cells (CFU) in the resuspended biofilm solution after contact with CHX, sodium hypochlorite (NaOCl) or plasma.

2.2. Antiseptic treatment

CHX digluconate (an extensively used antiseptic in dentistry) was used as a 0.1% aqueous solution. Firstly, the discs with the *C. albicans* biofilm were covered with 100 μl of the antiseptic and incubated for 1, 2, 5 or 10 min. Secondly, CHX was drawn off and the antiseptic effect was halted by adding 1 μl of an inactivator consisting of 40 g l^{-1} Tween 80, 30 g l^{-1} saponin,

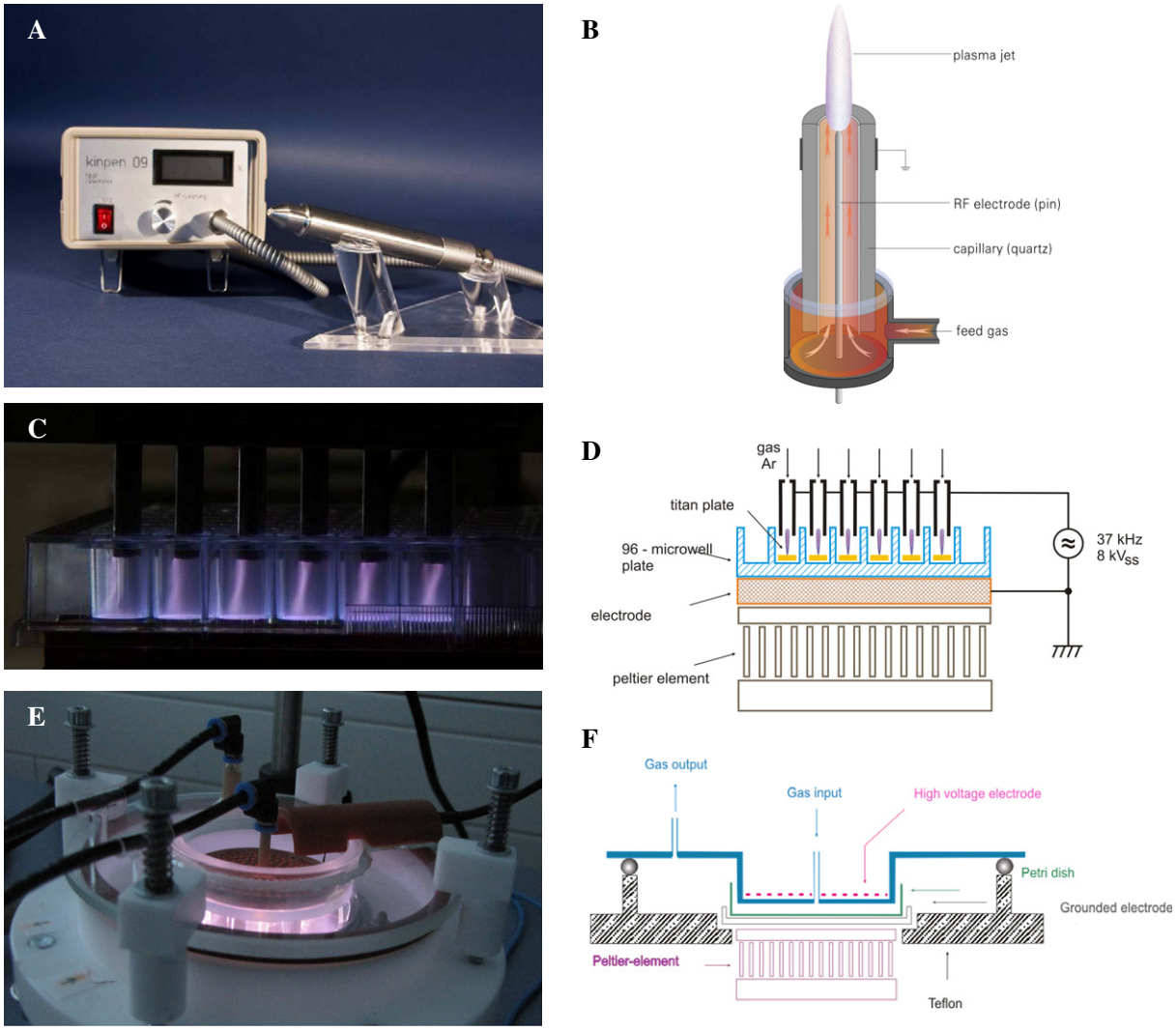

Figure 1. (A) Atmospheric pressure plasma jet (APPJ) kINPen09, (B) schematic setup of kINPen09, (C) HDBD: discharges in microtitre well plate, (D) schematic setup of HDBD, (E) VDBD reactor and (F) schematic setup of VDBD.

4 g l^{-1} lecithin, 10 g l^{-1} SDS and 1 g l^{-1} sodium thioglycolate. The inactivation of CHX by the inactivator was proven by the quantitative suspension test according to DIN EN 1040 (German Institute for Standardization).

Additionally, a 0.6% sodium hypochlorite solution (ApplyChem GmbH, Darmstadt, Germany) was used in the same way. An inactivator consisting of 30 g l^{-1} Tween 80, 3 g l^{-1} lecithin, 1 g l^{-1} histidin and 5 g l^{-1} sodium thiosulfate was used to halt the antifungal effect.

2.3. Plasma treatment

For plasma application, an APPJ 'kINPen09', a hollow electrode DBD and a volume DBD (figure 1), all developed by Leibniz Institute for Plasma Science and Technology (INP), Greifswald, Germany, were used.

2.3.1. KINPen09. The APPJ (kINPen® 09, INP Greifswald) described in this paper is shown in figure 1(A) [8]. The device has got the CE mark, which certifies that the product has met EU consumer safety, health or environmental requirements. The device consists of a hand-held unit (dimensions: length = 170 mm, diameter = 20 mm and weight = 170 g) for the generation of a plasma jet at atmospheric pressure, a dc power supply (system power: 8 W at 220 V, 50/60 Hz) and a gas supply unit. The principal scheme of the plasma source is shown in figure 1(B). At the centre of a quartz or ceramic capillary (inner diameter 1.6 mm), a pin-type electrode (1 mm diameter) is mounted. In the continuous working mode, a high-frequency (HF) voltage (1.82 MHz, 2–6 kV_{pp}) is coupled to the pin-type electrode. The plasma is generated from the top of the centred electrode and expands to the surrounding air outside the nozzle.

The whole system works with all rare gases (especially argon) with gas flow rates between 5 and 10 slm (standard litres per minute). Small admixtures ($\leqslant 1\%$) of molecular gases to the feed gas are possible. With these gas flow rates and a maximal input dc power of 3.5 W to the hand-held unit, the ignited plasma jet has a length of up to 12 mm. For our experiments the argon (Ar) gas flow was set to 5 slm. The flow rate was controlled by a flow controller (MKS Instruments, Munich, Germany). Temperature measurement resulted in 42 °C at the tip of the plasma jet, which is connected with a thermal output of 150 mW (input power 3 W). We chose a pin-to-sample distance of 7 mm, held constant during the application.

Generally, argon is the working gas for maintaining the discharge, while small amounts of oxygen can be admixed [8]. The plasma treatment is named 'Ar plasma' if no oxygen is admixed to the discharge and 'Ar + 1% O_2 plasma' if 1.0% (0.05 slm) oxygen is used as admixture, respectively.

Temperature measurement. Axial temperature profiles of the plasma jet were obtained by fibre optic temperature measurement (Luxtron, model 755, Santa Clara, USA). A temperature-dependent fluorescent signal of luminescent magnesium fluorogermanate was monitored, which was excited with an Xe flash lamp.

2.3.2. Dielectric barrier discharge. With the dielectric barrier discharge (DBD), at least one of the two metal electrodes is covered with a dielectric layer. Due to an inherent wall charge mechanism, the transition to an arc discharge is prevented. As a result, the discharge creates a non-thermal plasma without substantial heating of the gas.

2.3.2.1. Hollow electrode DBD. Here, another novel approach for the direct DBD treatment of microbiological samples is presented, the so-called hollow electrode DBD (HDBD; see figure 1(D) for a schematic view of the device). The system has been developed for the treatment of samples placed in wells of microtitre plates. Since microtitre plates are made of dielectric materials, they can serve as the barrier in a DBD arrangement. Therefore, microtitre plates were placed on the grounded electrode, which was cooled by a Peltier element in order to control the temperature of the objects to be treated. Six hollow and thin metal tubes served as high-voltage electrodes and gas injection pipes in one function. The gas flowed through these electrodes, while a high rf-voltage (37.6 kHz, 9 W, 9 kV) was coupled. Argon flow was set to 1 slm per well, which is equivalent to a total flow rate of 6 slm (1 slm through each of the six metal tubes). If oxygen was admixed, the total oxygen flow was set to 0.06 slm, which was equivalent to 0.01 slm per well. Two columns of the well plate remain for control samples. Figure 1(C) shows a photograph of the discharges in the microtitre plates.

The kINPen09 and the HDBD were clamped onto a computer-driven three-axis (x, y, z) motorized stage, under which a microtitre plate with the titanium discs was positioned. The discs were approached in succession and treated punctually according to the respective treatment period.

2.3.2.2. Volume DBD. Volume DBD (VDBD; see figure 1(E) for a photograph of the discharges and figure 1(F) for a schematic view of the device) consisted of two flat, round, metal electrodes, with one of them being electrically grounded. The high-voltage electrode was perforated to get a better insight into the discharge gap. A petri dish (Techno Plastic Products AG, Trasadingen, Switzerland) with the titanium discs was located between these electrodes. The bottom of the petri dish acted as the dielectric for the DBD. For cooling, a Peltier element was used. The gap (the distance between the disc and electrode) in this system was 15 mm and sealed air-tight. Argon gas (0.05 slm) flowed into the system. The high sinusoidal voltage (40 kHz, 16 W, 10 kV) applied between both electrodes generated the plasma. All eight samples were treated simultaneously.

There were two adjustable parameters for our plasma treatment: (i) time intervals of 1, 2, 5 and 10 min for each titanium sample and (ii) plasma gas composition.

Thus, two different gas compositions of plasma (in the case of VDBD, only argon was available) were applied for 1, 2, 5 or 10 min to the discs with the *C. albicans* biofilm. We repeated the treatment procedure eight times. Altogether, 160 discs were treated with three plasma devices (two gas admixtures × four treatment intervals and eight repetitions in the case of kINPen09 and HDBD; one gas admixture × four treatment intervals and eight repetitions in the case of VDBD).

To assess the effects of biofilm dehydration by gas flow (negative control), eight discs were treated with gas (argon or argon + 1% oxygen) for each treatment time (altogether again 160 discs). As a negative control for CHX and NaOCl treatment, we used 100 μl 0.9% NaCl solution (called NaCl control).

2.4. Scanning electron microscopy

For the electron microscopy, the biofilms were prepared as follows. After a fixation step (1 h in 1% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, 5 mM HEPES (pH 7.4) and 50 mM NaN_3), the samples were treated with 2% tannic acid for 1 h, 1% osmium tetroxide for 1 h, 1% thiocarbohydrazide for 30 min, 1% osmium tetroxide at 4 °C overnight and 2% uranyl acetate for 2 h with washing steps in between. The samples were dehydrated in a graded series of acetone solutions (10–100%) and then critical point dried. Finally, they were mounted on aluminium stubs, sputtered with gold–palladium and examined in an EVO LS10 (Zeiss, Oberkochen, Germany).

The preparation described is a typical protocol for the preparation of yeast cells for scanning electron microscopy. The first fixation step is necessary to fix the proteins in the cell. The following treatment—especially with osmium tetroxide—is used to stabilize the membranes. After fixation, the samples need to be dehydrated and dried because the examination occurs in the scanning electron microscope under high vacuum. Critical point drying with CO_2 is a very gentle method for sample drying because the critical point of CO_2 is in a range in which biological samples are not damaged.

From the authors' point of view, the preparation offsets the antiseptic and plasma-treated samples equally, because the damage occurred prior to processing the samples for electron microscopy. This preparation is intended to maintain the condition of the cells they had right before fixation.

2.5. Statistics

Continuous data are presented as means ± standard deviation.

Firstly, CFU were compared for each procedure and exposure time versus the NaCl control (treatment time 0 min) using the Mann–Whitney-*U* tests. Secondly, CFU values were compared for each procedure (kINPen09, HDBD and VDBD combined with gas/plasma compositions) versus the positive controls (CHX and NaOCl) within varying exposure times using one-sided Mann–Whitney-*U* tests. For each step, *P* values were corrected for multiple testing according to Benjamini and Hochberg [4]. Procedures (kINPen09, HDBD or VDBD with gas/plasma compositions) that did not reduce CFU values significantly compared to the control or the positive controls were excluded from further analyses.

Then, analysis of variance (ANOVA) and linear regression analyses were applied to evaluate differences in CFU values for different values of plasma sources (HDBD and VDBD combined with Ar gas, Ar plasma, Ar + 1% O_2 gas or Ar + 1% O_2 plasma) and treatment time (1, 2, 5 or 10 min). The twofold interaction term between both factors was considered and confirmed to be statistically significant ($P < 0.001$). *Post hoc* analyses of linear combinations of coefficients were carried out to examine differences in CFU reduction between different procedures and gas/plasma compositions.

Finally, for each exposure time, the best performing procedure was compared against the second best performing procedure applying *post hoc* Wald tests for linear hypotheses. Accordant *P* values for *post hoc* tests were adjusted for multiple testing [4]. Statistical differences were considered as significant at $P < 0.05$. Statistical analyses were performed with STATA/SE 10.0 (Stata Corp. LP, College Station, TX, USA).

3. Results

3.1. Positive controls and kINPen09

Firstly, 1 g (CFU ml^{-1}) values were compared for each procedure and exposure time versus controls. Except for three procedures (CHX and NaOCl, 1 min, and kINPen09 with Ar gas, 2 min), all procedures revealed a better antifungal effect ($P < 0.05$).

Secondly, the different plasma sources were compared against CHX and NaOCl within exposure times. CHX and NaOCl treatment of 1 min did not lead to a significant reduction in CFU. Between CHX, NaOCl and kINPen09 plasma (both gas compositions), a significant difference was not observed. At no time was treatment with CHX significantly superior to treatment with kINPen09. The results of NaOCl treatment were similar to those of CHX (CHX: $RF_{1\,min} = 0.3$, $RF_{2\,min} = 0.8$, $RF_{5\,min} = 0.9$, $RF_{10\,min} = 1.4$; NaOCl: $RF_{1\,min} = 0.2$, $RF_{2\,min} = 0.5$, $RF_{5\,min} = 0.7$, $RF_{10\,min} = 1.5$).

NaOCl-treated cells analyzed by scanning electron microscopy looked rugose (figure 4(B)). In contrast, CHX-treated cells were as undamaged as the NaCl controls. They seemed to be covered by a layer (figure 4(A)).

For kINPen09, CFU values were not consistently reduced compared to positive controls. The RFs were negligible (Ar plasma: $RF_{1\,min} = 0.4$, $RF_{2\,min} = 0.5$, $RF_{5\,min} = 0.5$, $RF_{10\,min} = 0.4$;

Table 1. Logarithm of *C. albicans* biofilm CFU ml^{-1} after treatment with different plasma devices combined with Ar plasma, Ar gas, Ar + 1% O_2 plasma or Ar + 1% O_2 gas for varying exposure times in comparison to NaCl controls and CHX/NaOCl-treated samples (positive controls). Mean lg(CFU ml^{-1}) ± S.D.

	Exposure time (min)				
	0	1	2	5	10
NaCl-control	7.54 ± 0.38				
CHX		7.27 ± 0.45	6.75 ± 0.36[a]	6.66 ± 0.33[a]	6.16 ± 0.59[a]
NaOCl		7.32 ± 0.15	7.02 ± 0.16[a]	6.84 ± 0.33[a]	6.08 ± 0.77[a]
kINPen09					
Argon gas		6.94 ± 0.47[a,c]	7.21 ± 0.42	6.85 ± 0.50[a]	7.01 ± 0.26[a]
Argon plasma		7.14 ± 0.15[a,c]	7.01 ± 0.30[a]	7.04 ± 0.14[a]	7.17 ± 0.22[a]
Argon + 1% O_2 gas		6.82 ± 0.49[a,c]	6.79 ± 0.33[a]	6.88 ± 0.49[a]	6.78 ± 0.44[a]
Argon + 1% O_2 plasma		6.92 ± 0.31[a,c]	6.51 ± 0.34[a,c]	6.73 ± 0.34[a]	6.99 ± 0.30[a]
HDBD					
Argon gas		6.60 ± 0.14[a,b,c]	6.45 ± 0.40[a,c]	6.64 ± 0.58[a]	6.20 ± 0.56[a]
Argon plasma		5.79 ± 0.37[a,b,c]	6.10 ± 0.37[a,b,c]	5.51 ± 0.29[a,b,c]	4.61 ± 0.42[a,b,c]
Argon + 1% O_2 gas		6.26 ± 0.36[a,b,c]	6.06 ± 0.49[a,b,c]	4.83 ± 0.33[a,b,c]	4.34 ± 0.41[a,b,c]
Argon + 1% O_2 plasma		6.11 ± 0.28[a,b,c]	6.13 ± 0.53[a,b,c]	4.45 ± 0.39[a,b,c]	4.22 ± 0.56[a,b,c]
VDBD					
Argon gas		5.72 ± 0.96[a,b,c]	5.58 ± 0.51[a,b,c]	5.50 ± 0.81[a,b,c]	6.23 ± 0.72[a]
Argon plasma		5.17 ± 0.69[a,b,c]	5.32 ± 1.16[a,b,c]	3.98 ± 0.63[a,b,c]	2.32 ± 1.06[a,b,c]

[a] $P < 0.05$ versus control (0 min).
[b] $P < 0.05$ versus CHX within the same exposure times, one-sided Mann–Whitney-U-test.
[c] $P < 0.05$ versus NaOCl within the same exposure times, one-sided Mann–Whitney-U-test.

Ar + 1% O_2 plasma: $RF_{1\,min} = 0.6$, $RF_{2\,min} = 1.0$, $RF_{5\,min} = 0.8$ and $RF_{10\,min} = 0.6$). For exposure times of 10 min, CFU values were even significantly higher after kINPen09 treatment (Ar plasma 7.17 ± 0.26; Ar + 1% O_2 6.99 ± 0.30) compared to CHX (6.16 ± 0.59)-treated samples ($P < 0.05$). Consequently, kINPen09 was omitted from further analyses.

Using scanning electron microscopy, we found areas with many intact cells (figure 3(A)), which look exactly like the NaCl control (data not shown). Only in a limited area did we detect damaged cells.

3.2. Dielectric barrier discharges (DBDs)

All DBD procedures revealed better antifungal effects ($P < 0.05$) than the NaCl controls. Overall, for HDBD and VDBD, reduction in CFU was significantly higher compared to positive controls (CHX and NaOCl; $P < 0.05$).

After 10 min, the three HDBD groups (Ar plasma, Ar + 1% O_2 gas and Ar + 1% O_2 plasma) reduced the CFU ml^{-1} by a $\log_{10}$ RF of 2.9, 3.2 and 3.3, respectively, while CHX only achieved a reduction factor of 1.5 (table 1). In the case of VDBD, the CFUs were reduced by a $\log_{10}$ RF of 2.3 for 1 min, 2.2 for 2 min, 3.5 for 5 min and 5.2 for 10 min of plasma treatment.

Table 2. ANOVA and according linear regression models evaluating effects of HDBD and VDBD combined with Ar plasma, Ar gas, Ar + 1% O_2 plasma or Ar + 1% O_2 gas and exposure time on CFU. $R^2 = 76.6\%$.

	Linear regression		ANOVA	
	B (95% CI)	*P* value	R^2	*P* value
Time (ref.: 1 min)				
2 min	−0.16 (−0.75; 0.42)	0.60		
5 min	0.04 (−0.55; 0.63)	0.90		
10 min	−0.41 (−1.00; 0.18)	0.17	22.8%	<0.001
Method (ref.: HDBD Ar gas)				
HDBD Ar plasma	−0.81 (−1.40; −0.22)	0.007		
HDBD Ar + 1% O_2 gas	−0.35 (−0.94; 0.24)	0.24		
HDBD Ar + 1% O_2 plasma	−0.49 (−1.08; 0.10)	0.10		
VDBD Ar gas	−0.89 (−1.47; −0.30)	0.003		
VDBD Ar plasma	−1.43 (−2.02; −0.85)	<0.001	34.7%	<0.001
Method × time				
HDBD Ar plasma, 2 min	0.46 (−0.37; 1.30)	0.27		
HDBD Ar plasma, 5 min	−0.33 (−1.16; 0.50)	0.44		
HDBD Ar plasma, 10 min	−0.78 (−1.61; 0.05)	0.066		
HDBD Ar + 1% O_2 gas, 2 min	−0.03 (−0.87; 0.80)	0.94		
HDBD Ar + 1% O_2 gas, 5 min	−1.47 (−2.30; −0.63)	0.001		
HDBD Ar + 1% O_2 gas, 10 min	−1.50 (−2.34; −0.67)	<0.001		
HDBD Ar + 1% O_2 plasma, 2 min	0.18 (−0.66; 1.01)	0.68		
HDBD Ar + 1% O_2 plasma, 5 min	−1.70 (−2.53; −0.87)	<0.001		
HDBD Ar + 1% O_2 plasma, 10 min	−1.48 (−2.32; −0.65)	0.001		
VDBD Ar gas, 2 min	0.02 (−0.81; 0.86)	0.95		
VDBD Ar gas, 5 min	−0.25 (−1.09; 0.58)	0.55		
VDBD Ar gas, 10 min	0.92 (0.09; 1.75)	0.03		
VDBD Ar plasma, 2 min	0.31 (−0.52; 1.14)	0.47		
VDBD Ar plasma, 5 min	−1.23 (−2.06; −0.40)	0.004		
VDBD Ar plasma, 10 min	−2.44 (−3.27; −1.61)	<0.001	19.1%	<0.001
Constant	6.60 (6.19; 7.02)	<0.001		

To evaluate the impact of method and exposure time on CFU, ANOVAs with accordant linear regression models were computed (table 2). According to the ANOVA, 34.7 and 22.8% of the variation in CFU values could be explained by the method and exposure time, respectively, whereas 19.1% of the variation was explained by the interaction between both factors ($P < 0.001$). Overall, the model explains 76.6% of the total variance. After 1 min, CFUs were significantly reduced for HDBD Ar plasma, VDBD Ar gas and VDBD Ar plasma compared to HDBD Ar gas ($P = 0.007$, $P = 0.003$ and $P < 0.001$, respectively). Increased exposure time (i.e. 5 or 10 min) further reduced CFU values significantly for HDBD Ar + 1% O_2 gas, HDBD Ar + 1% O_2 plasma, VDBD Ar gas (only for 5 min) and VDBD Ar plasma.

Post hoc analyses of linear combinations of coefficients (table 3 and figure 2) revealed that, compared to HDBD Ar gas 1 min, CFU values were significantly reduced for VDBD Ar gas (all times, except 10 min), VDBD Ar plasma (all times), HDBD combined with Ar plasma with

Table 3. Linear combinations of coefficients for the regression model in table 2. Point estimates with their 95% confidence intervals (CI) are given.

	1 min	2 min	5 min	10 min
HDBD Ar gas	0	−0.16 (−0.75; 0.43)	0.04 (−0.55; 0.63)	−0.41 (−1.00; 0.18)
HDBD Ar plasma	−0.81 (−1.40; −0.22)[b]	−0.50 (−1.09; 0.08)	−1.10 (−1.69; −0.51)[c]	−2.00 (−2.59; −1.41)[c]
HDBD Ar + 1% O_2 gas	−0.35 (−0.94; 0.24)	−0.54 (−1.13; 0.05)	−1.78 (−2.36; −1.19)[c]	−2.26 (−2.85; −1.67)[c]
HDBD Ar + 1% O_2 plasma	−0.49 (−1.08; 0.10)	−0.47 (−1.06; 0.11)	−2.15 (−2.74; −1.57)[c]	−2.70 (−3.54; −1.87)[c]
VDBD gas	−0.89 (−1.47; −0.30)[b]	−1.02 (−1.61; −0.43)[b]	−1.10 (−1.69; −0.51)[c]	−0.37 (−0.96; 0.21)
VDBD plasma	**−1.43 (−2.02; −0.85)[c]**	**−1.28 (−1.87; −0.70)[c]**	**−2.63 (−3.21; −2.04)[c]**	**−4.29 (−4.87; −3.70)[c]**
Wald test VDBD plasma versus				
HDBD Ar gas	<0.001	0.0004	<0.001	<0.001
HDBD Ar plasma	0.045	0.0129	<0.001	<0.001
HDBD Ar + 1% O_2 gas	0.0007	0.017	0.0075	<0.001
HDBD Ar + 1% O_2 plasma	0.0032	0.010	0.12	<0.001
VDBD Ar gas	0.075	0.38	<0.001	<0.001

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

treatment times of 2, 5 or 10 min ($P < 0.05$), and HDBD combined with Ar + 1% O_2 gas or Ar + 1% O_2 plasma with treatment times of 5 or 10 min ($P < 0.05$).

To compare CFU values for VDBD plasma with the procedure with the second best performance within exposure times, we applied *post hoc* Wald tests for linear hypotheses (adjusted for multiple testing). They revealed that VDBD combined with plasma was the significantly best performing procedure for an exposure time of 10 min among all procedures ($B = -4.29$ (-4.87; -3.70), $P < 0.05$).

SEM micrographs of 10 min HDBD-treated cells revealed massive perforations to cell walls. Most cells seemed to be evenly split (figure 4(C)). SEM micrographs of 10 min VDBD-treated cells were similar to those of HDBD (figure 4(D)). In contrast to kINPen09-treated cells, only small areas of intact cells could be examined under the scanning electron microscope.

4. Discussion

The presence of *C. albicans* is crucial in the etiology of prothesis stomatitis and is reported to be found often on surfaces of acrylic resin materials *in vivo*. It has been, furthermore, repeatedly isolated from infected peri-implant sites (inflammatory tissue surrounding dental implants) [20]. Those kinds of yeast infections are difficult to treat with topical measures, so new methods need to be developed for effective treatment [17].

Since denture brushing is seldom performed as a daily routine by elderly people with acceptable efficacy, and treatment with ultrasonic baths is not effective, chemical cleansing with immersion baths is the first choice for denture plaque control [12]. Many cleansers have been commercialized. Among alkaline peroxides, CHX gluconate and NaOCl, NaOCl is considered as the most effective and CHX as the least effective against adhered *C. albicans* cells [11]. New approaches with enhanced efficacy are therefore highly appreciated [31].

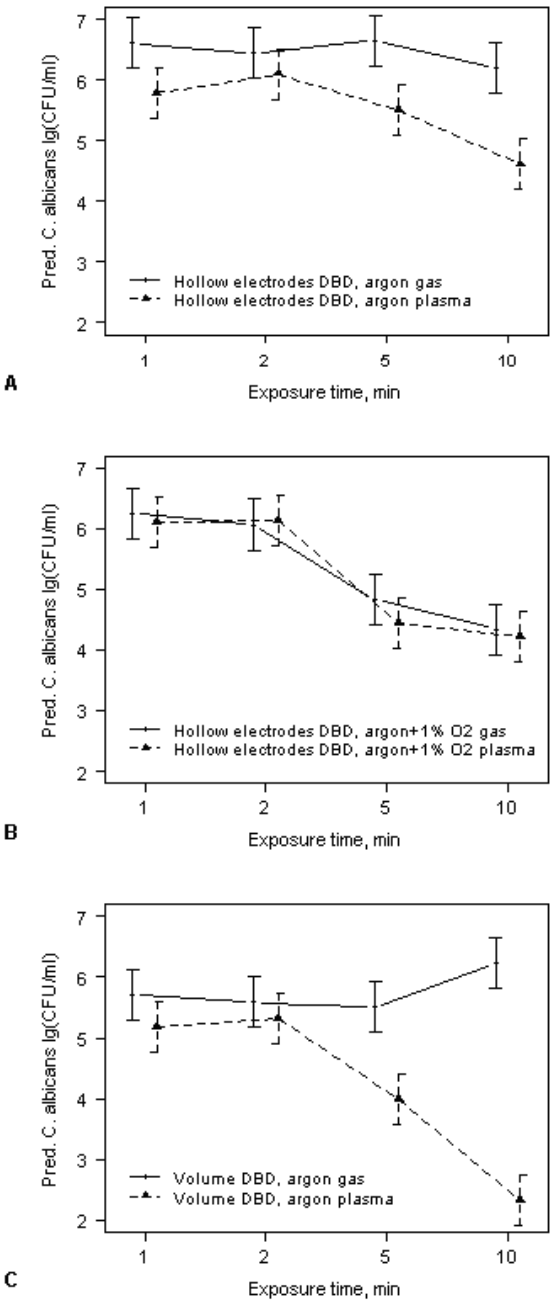

Figure 2. Predicted (Pred.) logarithm of *C. albicans* biofilm CFU ml^{-1} after treatment for different exposure times with (A) HDBD, Ar gas/plasma; (B) HDBD, Ar + 1% O_2 gas/plasma; (C) VDBD Ar gas/plasma.

Here, we have demonstrated that DBD plasma from different sources was even more effective in the treatment of *C. albicans* biofilms when compared to standard procedures.

Our study had several limitations. We used an *in vitro* model to compare the efficacy of different plasma devices with chemical cleansing. To ensure reproducibility of the results, a standard method of biofilm research was used. For a better understanding of the antifungal effects of plasma treatment, we performed our experiments on titanium discs to exclude material hydrophobicity and retention niches, such as cavities and porosities into which the biofilm

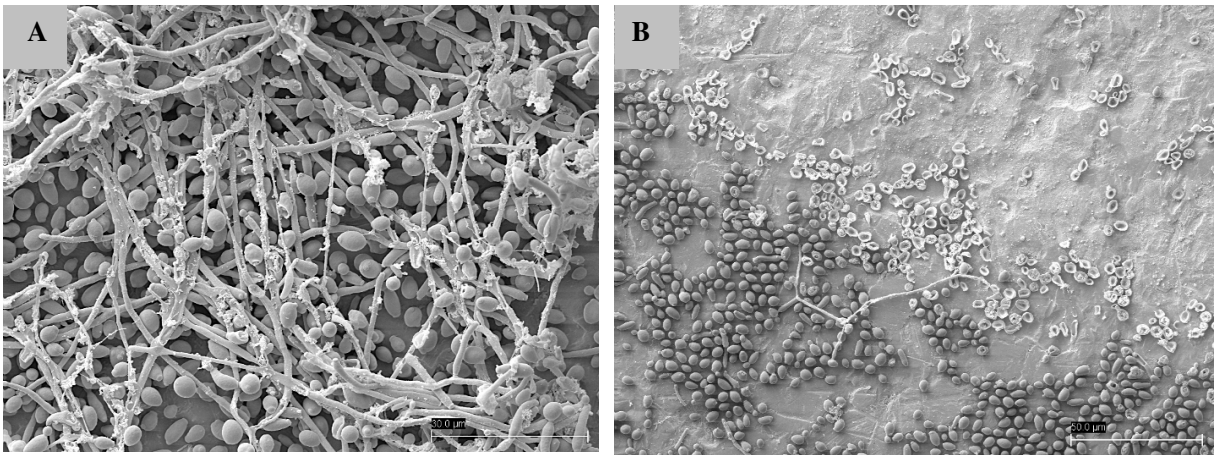

Figure 3. (A) *C. albicans* biofilm on kINPen09 plasma-treated discs outside the treatment area, magnification 1000fold. (B) Destroyed biofilm on the clear boundary of the treatment area with damaged and intact *C. albicans* cells, magnification 500fold.

could adhere. Furthermore, we wanted to exclude putative chemical interactions of residual monomers or of surface properties with adhesion of *C. albicans*. Future experimental series will be performed on denture resin discs to better simulate the oral environment. Since other groups showed that *C. albicans* is a common colonizer of infected dental implants [20], our results may also be applicable to implant dentistry and may help to develop future treatment protocols against the peri-implant biofilms.

The plasma effects against *C. albicans* biofilms *in vitro* were compared with those of CHX and NaOCl, representing chemical cleansers [11, 38]. CHX is a frequently used antiseptic [18, 37]. But, depending on the test system, CHX was proven to be ineffective against biofilms [35]. The efficacy of CHX depends on its concentration and particularly on its exposure time. It also has a fungistatic effect on yeasts [1]. Our results are in agreement with Gedik and Özkan [11], who showed only a moderate effect of CHX despite 90 min immersion in a cleansing bath. NaOCl has been used for over 200 years for disinfection purposes and is one of the most effective substances currently used in dentistry for biofilm treatment [33].

The antimicrobial mechanism of plasma is more complex than that of CHX. CHX attacks all cell membranes, which are dissolved and the cytoplasm leaks out. Plasma affects many cell components (especially proteins, lipids and DNA/RNA) [7]. Disinfection with plasma is based on short-lived active species, radicals (reactive oxygen species, ROS) and UV radiation. ROS can cause lipid peroxidation. As a result, the membrane is damaged and the cell lyses [7]. Electrostatic effects of the electric field have an additional antimicrobial effect [23]. Inactivation, bio-decontamination and sterilization have received much attention in recent years [9]. Plasma is known to inactivate planktonic bacteria, yeasts and spores [10, 17, 28, 34]. Morfill *et al* [24] reduced 4 $\log_{10}$ of *C. albicans* on agar plates after 5 s plasma treatment. Using a plasma pen, a 3–4 $\log_{10}$ reduction in adherent bacteria and in *C. albicans (*non-biofilm) has been demonstrated [29]. In contrast, a $\log_{10}$ reduction factor of only 1.5 of a 24-h-old *Pantoea agglomerans* biofilm was achieved (plasma pen with 5 slm He and 0.01% O_2) [36]. Our results are in agreement with these findings. We also observed a minor $\log_{10}$ reduction factor of only 1 with kINPen09 with similar settings on mature biofilms. Regarding our SEM images (figures 3

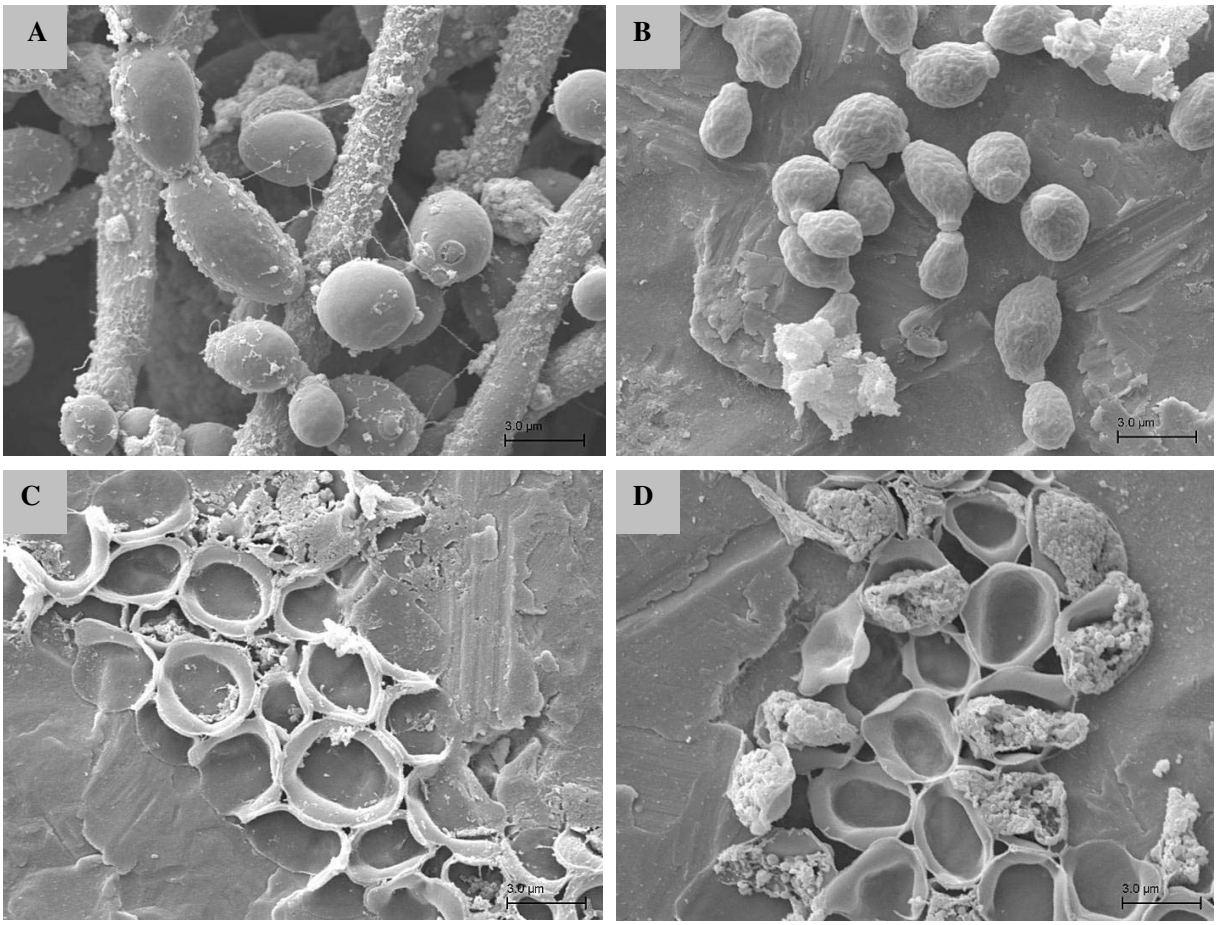

Figure 4. *C. albicans* biofilms on titanium discs after treatment with (A) CHX digluconate, (B) with sodium hypochloride (leading to rugose yeast cells), (C) with HDBD (leading to completely destroyed cells) and (D) with VDBD (leading to completely destroyed cells). Magnification 5000fold.

and 4), plasma could remove 48-h-old *C. albicans* biofilms. Lee *et al* [19] showed similar effects, but only on 24-h-old bacterial biofilms. Comparing the different plasmas used in our study, the antifungal efficacy of DBD plasma (VDBD and HDBD) was significantly better compared to kINPen09 ($P < 0.05$). One reason for these minimal antifungal effects of kINPen09 could be that the spot treatments with the narrow plasma afterglow damage only yeast cells at the actual touch point and that the reactive plasma species, etc do not spread over the entire disc in a sufficient dose. Analyses by scanning electron microscopy showed clear boundaries of damaged cells underlining this hypothesis (figure 3(B)). Therefore, in further experiments, the pen should be moved all over the disc, instead of treating only one area. Increased exposure time of plasma treatment showed no effects for kINPen09.

Compared to kINPen09, the DBD plasma achieved a $\log_{10}$ reduction factor between 3.1 (HDBD) and 5.2 (VDBD). After DBD plasma treatment, single cells looked exactly like those treated with kINPen09 (figure 4C). We also observed boundaries between treated and untreated or not sufficiently treated areas. Thus, most cells were severely damaged by DBD plasma treatment. Only in small areas did cells look intact.

In a plasma process, a high gas stream is necessary to keep the plasma cool [3]. In comparison with the NaCl control discs, a gas stream has a certain antifungal effect. Antimicrobial effects of irrigation with non-inert gases were shown *in vivo* [30]. DBD plasma processes allow the use of a low gas flow. We have admixed oxygen to provide the necessary oxygen species. Treatment with flowing argon + 1% O_2 gas resulted in higher reductions than treatment with pure argon gas. The reduction factor increased with increasing exposure time, probably because of oxidative stress. DBD plasma consists of many microstreamers that last only a few nanoseconds [21]. The disadvantage of DBD plasma is that it is not homogeneous throughout the volume. However, the whole disc was treated in contrast to kINPen09. Thus, some regions of an intact biofilm stayed undamaged, probably due to the inhomogeneities of the plasma. The DBD plasma was subdivided into HDBD and VDBD. VDBD plasma was significantly the best procedure for an exposure time of 10 min among all procedures. While the VDBD worked with 16 W, 40 kHz and 10 kV, the HDBD worked with 9 W, 37.6 kHz and 9 kV. Hence, at first sight, the higher input power of the VDBD seems to be the reason for the increased reduction factor of VDBD (5.2 after 10 min) in contrast to HDBD (3.1) after 10 min. On the other hand, both measured powers are system powers and did not equal the real power injected into the plasma. Therefore, a direct comparison of both plasmas is difficult. For this purpose, further diagnostics are necessary. After the first 2 min, the $\log_{10}$ reduction factor was the same for both devices. After 5 min treatment with HDBD, Ar + 1% O_2 plasma was the second best procedure.

In contrast to kINPen09 plasma, biofilms treated with DBD plasma were located between electrodes in the middle of an electric field. The disc was the counterelectrode, so that the electric current went through the biofilm. The increased electric field strength could be another reason for better inactivation of *C. albicans* by both DBDs in comparison with kINPen09. This property, and the possibility of treating a whole denture with plasma, are ideal for denture cleaning. Therefore, atmospheric pressure plasma may become an alternative to chemical cleansing.

5. Conclusions

C. albicans biofilms play a key role in the pathogenesis of denture stomatitis. CHX and NaOCl were shown to be only moderately effective against yeast cells in biofilms. In this study, the effect of three different plasma devices on *C. albicans* biofilms in comparison with CHX and NaOCl was investigated. We found that antifungal therapy with nonthermal plasma is effective against biofilms. DBD plasmas exceed the antifungal effects of CHX and NaOCl. Further research is going on to evaluate the cause of the different efficacies and the possible role of physical therapies for biofilm-derived infections in dental practice.

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References

[1] Barasch A, Safford M M, Dapkute-Marcus I and Fine D H 2004 Efficacy of chlorhexidine gluconate rinse for treatment and prevention of oral candidiasis in HIV-infected children: a pilot study *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **97** 204–7

[2] Barbeau J, Seguin J, Goulet J P, de Koninck L, Avon S L, Lalonde B, Rompre P and Deslauriers N 2003 Reassessing the presence of *Candida albicans* in denture-related stomatitis *Oral Surg. Oral Med. O* **95** 51–9

[3] Bender C, Matthes R, Kindel E, Kramer A, Lademann J, Weltmann K D, Eisenbeiß W and Hübner N O 2010 The irritation potential of nonthermal atmospheric pressure plasma in the HET-CAM *Plasma Process. Polym.* **7** 318–26

[4] Benjamini Y and Hochberg Y 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing *J. R. Stat. Soc.* B **57** 289–300

[5] da Costa K R C, Ferreira J C, Komesu M C and Candido R C 2009 *Candida albicans* and *Candida tropicalis* in oral candidosis: quantitative analysis, exoenzyme activity, and antifungal drug sensitivity *Mycopathologia* **167** 73–9

[6] Dar-Odeh N S and Shehabi A A 2003 Oral candidosis in patients with removable dentures *Mycoses* **46** 187–91

[7] Filoche S K, Sissons C, Sladek R E J and Stoffels E 2008 *Plasma Assisted Decontamination of Biological and Chemical Agents* ed S Güceri and A Fridman (Dordrecht: Springer) pp 65–78

[8] Foest R, Kindel E, Ohl A, Stieber M and Weltmann K D 2005 Non-thermal atmospheric pressure discharges for surface modification *Plasma Phys. Control Fusion* **47** B525–36

[9] Foest R, Schmidt M and Becker K 2006 Microplasmas, an emerging field of low-temperature plasma science and technology *Int. J. Mass Spectrom.* **248** 87–102

[10] Fridman G, Brooks A D, Balasubramanian M, Fridman A, Gutsol A, Vasilets V N, Ayan H and Friedman G 2007 Comparison of direct and indirect effects of non-thermal atmospheric-pressure plasma on bacteria *Plasma Process. Polym.* **4** 370–5

[11] Gedik H and Özkan Y K 2009 Cleaning efficiency of alkaline peroxide type denture cleansers on silicone-based soft lining materials colonized with *Candida albicans Balk. J. Stom.* **13** 35–40

[12] Gedik H and Özkan Y K 2009 The effect of surface roughness of silicone-based resilient liner materials on the adherence of *Candida albicans* and inhibition of *Candida albicans* with different disinfectants *Oral Health Prev. Dent.* **7** 347–53

[13] Hawser S P and Douglas L J 1995 Resistance of *Candida albicans* biofilms to antifungal agents *in vitro Antimicrob. Agents Chemother.* **39** 2128–31

[14] He M, Du M, Fan M and Bian Z 2007 *In vitro* activity of eugenol against *Candida albicans* biofilms *Mycopathologia* **163** 137–43

[15] Keng S B and Lim M 1996 Denture plaque distribution and the effectiveness of a perborate-containing denture cleanser *Quintessence Int.* **27** 341–5

[16] Koban I, Hübner N O, Matthes R, Welk A, Kindel E, Weltmann K D, Kramer A and Kocher T 2009 Antiseptische Wirksamkeit ausgewählter Wirkstoffe und von tissue tolerable plasma (TTP) auf *C. albicans*-Biofilme – hat der Reifegrad des Biofilms einen Einfluss? *GMS Krankenhaushyg. Interdiszip.* **4** Doc09

[17] Kolb J F, Mohamed A A H, Price R O, Swanson R J, Bowman A, Chiavarini R L, Stacey M and Schoenbach K H 2008 Cold atmospheric pressure air plasma jet for medical applications *Appl. Phys. Lett.* **92** 241501–3

[18] Lamfon H, Porter S R, McCullough M and Pratten J 2004 Susceptibility of *Candida albicans* biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study *J. Antimicrob. Chemother.* **53** 383–5

[19] Lee M H, Park B J, Jin S C, Kim D, Han I, Kim J, Hyun S O, Chung K H and Park J C 2009 Removal and sterilization of biofilms and planktonic bacteria by microwave-induced argon plasma at atmospheric pressure *New J. Phys.* **11** 115022

[20] Leonhardt A, Bergström C and Lekholm U 2003 Microbiologic diagnostics at titanium implants *Clin. Implant Dent. Relat. Res.* **5** 226–32

[21] Liu C, Cui N, Brown N M D and Meenan B J 2004 Effects of DBD plasma operating parameters on the polymer surface modification *Surface Coat. Technol.* **185** 311–20
[22] Lombardi T and Budtz-Jörgensen E 1993 Treatment of denture-induced stomatitis: a review *Eur. J. Prosthodont. Restor. Dent.* **2** 17–22
[23] Morfill G E, Kong M G and Zimmermann J L 2009 Focus on plasma medicine *New J. Phys.* **11** 115011
[24] Morfill G E, Shimizu T, Steffes B and Schmidt H U 2009 Nosocomial infections–a new approach towards preventive medicine using plasmas *New J. Phys.* **11** 115019
[25] Müller G, Winkler Y and Kramer A 2003 Antibacterial activity and endotoxin-binding capacity of Actisorb Silver 220 *J. Hosp. Infect.* **53** 211–4
[26] Nakamoto K, Tamamoto M and Hamada T 1991 Evaluation of denture cleansers with and without enzymes against *Candida albicans J. Prosthet. Dent.* **66** 792–5
[27] Pompl R *et al* 2009 The effect of low-temperature plasma on bacteria as observed by repeated AFM imaging *New J. Phys.* **11** 115023
[28] Rupf S, Hannig M, Schäfer B, Schubert A, Lehmann A C, Hofmann T and Schindler A 2009 Antimicrobial activity of non-thermal atmospheric plasma jet *Caries Res.* **43**
[29] Rupf S, Lehmann A, Hannig M, Schafer B, Schubert A, Feldmann U and Schindler A 2010 Killing of adherent oral microbes by a non-thermal atmospheric plasma jet *J. Med. Microbiol.* **59** 206–12
[30] Schlagenhauf U, Horlacher V, Netuschil L and Brecx M 1994 Repeated subgingival oxygen irrigations in untreated periodontal patients *J. Clinic. Periodont.* **21** 48–50
[31] Sennhenn-Kirchner S, Schwarz P, Schliephake H, Konietschke F, Brunner E and Borg-Von Zepelin M 2008 Decontamination efficacy of erbium:yttrium–aluminium–garnet and diode laser light on oral *Candida albicans* isolates of a 5-day in vitro biofilm model *Laser Med. Sci.* **24** 313–20
[32] Sladek R E J and Stoffels E 2005 Deactivation of *Escherichia coli* by the plasma needle *J. Phys. D: Appl. Phys.* **38** 1716–21
[33] Spratt D A, Pratten J, Wilson M and Gulabivala K 2001 An *in vitro* evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates *Int. Endod. J.* **34** 300–7
[34] Woedtke V T, Kramer A and Weltmann K D 2008 Plasma sterilization: what are the conditions to meet this claim? *Plasma Process. Polym.* **5** 534–9
[35] Vitkov L, Hermann A, Krautgartner W, Herrmann M, Fuchs K, Klappacher M and Hannig M 2005 Chlorhexidine-induced ultrastructural alterations in oral biofilm *Microsc. Res. Tech.* **68** 85–9
[36] Vleugels M, Shama G, Deng X T, Greenacre E, Brocklehurst T and Kong M G 2005 Atmospheric plasma inactivation of biofilm-forming bacteria for food safety control *IEEE Trans. Plasma Sci.* **33** 824–8
[37] Wirthlin M, Chen P and Hoover C 2005 A laboratory model biofilm fermenter: design and initial trial on a single species biofilm *J. Periodont.* **76** 1443–9
[38] Zelic O, Cakic S and Lukovic N 2009 The effect of two different oral antiseptics on dental plaque formation (*de novo* biofilm) and on gingival inflammation *Srp. Arh. Celok. Lek.* **137** 6–9