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Ex Vivo Study Comparing Three Cold Atmospheric Plasma (CAP) Sources for Biofilm Removal on Microstructured Titanium

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ABSTRACT: The aim of the present experimental study was to test three different plasma sources on the removal of 72 h oral biofilms. It was hypothesized that cold atmospheric plasma (CAP) lowers biofilm coverage significantly. *In vivo* biofilms were formed on sand-blasted/acid-etched titanium discs (n = 40) mounted on splints worn for 72 h by eight volunteers. Specimens were randomly divided into five groups: CAP I received indirect plasma application, CAP II received direct plasma application, CAP III received microwave-driven pulsed plasma application (90 s each). The chlorhexidine (CHX) group was cleaned with a curette and rinsed with CHX. Biofilms of a control group received no treatment. After treatment, all specimens were rinsed for 10 s using a dental air/water spray (2 bar). The vitality of microorganisms was detected by cultivation on agar plates for 24 h and 48 h. The presence of biofilms and their quantity on the titanium samples was investigated by fluorescence microscopy (FM) using live/dead staining. A biofilm's quality was analyzed by scanning electron microscopy (SEM). All treated samples showed a reduced growth on agar plates compared with the control group. FM analysis showed significantly lowered biofilm coverage in all treatment groups compared with the negative control group (t test, $p < 0.05$). Within the plasma treatment groups, there was a significant difference between CAP II and CAP III ($p = 0.032$). SEM showed disintegrated biofilms in all test groups. CAP reduces and disintegrates oral biofilms. Adjuvant application plasma could lead to more effective antimicrobial therapies for peri-implantitis.

KEY WORDS: cold atmospheric plasma, peri-implantitis, direct plasma, indirect plasma

I. INTRODUCTION

Plasma is an ionized gas and is referred to as the fourth state of matter.[1,2] Plasma consists of various portions of electrons, neutrons, and ionized atoms, as well as neutral or charged molecules. It can be generated under normal pressure conditions in so-called plasma jet sources by adding energy in the form of heat, microwaves, radio frequency, or pulsed direct current high voltage to a gas stream.[2]

Its various "effect-cocktail" on biological material or tissues is due to charged particles, chemically reactive species such as reactive oxygen species and reactive nitrogen species, heat, and UV radiation.[1,3–5] The current therapeutic application spectrum of plasma in medicine reaches from well-established sterilization of surgical instru-

ments, implants, and consumables, coagulation, ablation, and vaporization of tissues to its more recent utilization as antimicrobial therapy, stimulation of wound healing, and tumor therapy.[1,6–10] Exposure to cold atmospheric plasma (CAP) effectively leads to inactivation and destruction of a variety of even multi-resistant bacteria and bacterial biofilms, as wells as spores, fungi, and viruses.[8,9,11–17] To date, no resistance against plasma is known.[9,18,19]

Worldwide, the number of inserted dental implants used as an established therapy for missing teeth is increasing.[20] In many cases, dental implants are the only way to restore missing molar teeth and toothless jaws in a fixed prosthetic manner.[21]

Peri-implant diseases are among the most common complications after dental implantation.[20,22] Due to their clinical phenotype, peri-implant diseases are categorized in reversible inflammatory reactions in the soft tissues as mucositis and irreversible, progressive inflammatory reactions with loss of supporting bone as peri-implantitis.[21–24] Up to 56% of inserted dental implants develop peri-implantitis.[25] Until now, there has been no ideal therapy for these cases.[20,21]

The etiological factor for the development of peri-implant inflammations is the formation of oral biofilms on the surface of intraosseus dental implants and on their supraconstructions such as crowns, which are exposed to the oral environment.[21,24] Biofilms are formed as microbial communities on the interface of hard surface and biological liquids and consist of bacteria, extracellular polysaccharid matrix, proteins, and nuclein acids. The development and maturation of oral biofilms proceeds in different stages.[26,27] Within minutes, a so-called pellicle layer is formed out of adsorbed salivary proteins, glycoproteins, and mucin. In the next minutes to hours, bacteria adhere on the pellicle's surface, divide, and recruit more planktonic bacterial cells. A three-dimensional multilayer biofilm is formed by bacterial growth, co-adherence of further bacterial species, and maturation of the extracellular matrix due to bacterial glycosyltransferases synthesizing water-soluble and -insoluble glucans.[26]

The exceptional biocompatibility of titanium as a dental implant material makes it ideal for osteoblast and fibroblast adsorption, but also for biomolecular pellicles and thus accumulation of microorganisms on their surfaces.[27,28] Without therapy, bone degeneration caused by peri-implantitis can lead to implant loss and may have further systemic effects on the cardiovascular system due to bacteria reaching the bloodstream.[20,21,29,30]

Traditional therapies aim at the destruction of oral biofilms on infected implant surfaces.[20,21] Conservative, non-surgical therapy options are mechanical cleaning, smoothing, and polishing with curettes of different materials (Teflon, carbon, plastic, titanium), ultrasonic systems, air-polishing with water-soluble powders or laser treatment, often in combination with antimicrobial agents or antibiotics.[20,21,31] However, none of these therapeutic procedures are able to arrest peri-implantitis. Apart from maximum invasive explantation, which also causes large bone defects that often make re-implantation impossible, no reliable therapy for peri-implant diseases is available to date.[20,21,23] Accordingly, there is a great technical and therapeutical interest in methods for the decontamination of titanium surfaces.

The novel field of plasma medicine and the local application of CAP could offer an attractive clinical concept for inactivation and destruction of adherent bacteria and biofilm and its capability in this regard has been shown in several studies.[8,9,12–14,16,17,19] Using a sequential plasma–water spray–plasma treatment, it was possible to remove oral 72 h biofilms completely while preserving the titanium microstructure.[32,33]

The aim of this experimental study was to compare three different plasma sources for removal of 72 h biofilms formed *in situ* from the microstructure of sand-blasted/acid-etched titanium dental implant surfaces. It was hypothesized that using CAP and water spray in a simplified two-step sequence leads to significant lower biofilm coverage on titanium.

II. MATERIALS AND METHODS

A. *In Vivo* Biofilm Development

Eight healthy volunteers (four male, four female, mean age: 26.8 years) wore individual, custom-made maxillary plastic splints with five micro-structured titanium specimens (Dentsply Implants, Mannheim, Germany) placed on the buccal sides of the premolar and molar teeth for 72 h. The titanium discs ($n = 40$, sand-blasted, acid-etched, titanium grade 2, d: 5 mm, h: 1 mm, mean roughness: 1.96 μm) were fixed with silicone impression material (President light body, Coltene, Switzerland) on the splints for intraoral biofilm formation.[8] The study protocol was approved by the ethical committee of Charité Universitätsmedizin Berlin (EA4/090/017) and written informed consent was obtained from all participants.

During the 72 h exposure time, the volunteers followed their usual drinking and eating habits, but avoided biofilm-formation-inhibiting products such as tea and red wine. No mechanical cleaning or chemical products for plaque control such as toothpaste or mouth rinses were used. Splints were removed for meals or drinking only and then stored in a humid compartment (water-soaked cellulose fabric in an oxygen-proof plastic container).

After biofilm formation, the titanium discs were placed in a humid container such as described above and stored in a cool environment (7°C) until further treatment.

B. Anti-Biofilm Treatment

The titanium discs covered with 72 h *in situ* biofilms were rinsed *ex vivo* with sterile saline solution (0.9%) for 10 s. Titanium discs from each volunteer were randomly divided into five groups of eight specimens each. Each treatment consisted of two steps: (1) the plasma or mechano-antimicrobial procedure and (2) air–water spraying.

1. Plasma Treatment and Mechano-Antimicrobial Procedure

Indirect CAP (CAP I) was applied for 90 s by constantly moving the plasma jet (kINPen® MED, Neoplas Tools GmbH, Greifswald, Germany[25,26]), fed with 4.3 $sL*min^{-1}$

argon in a perpendicular position and within approximately 3 mm distance from the specimens' surface.

Direct plasma (CAP II) was generated using the PlasmaDerm FLEX 9060 (Cinogy, Duderstadt, Germany). Duration of voltage pulses was 10 µs at amplitudes up to 10 kV and a repetition rate of 300 Hz. A mean input power was defined as 450 mW according to the manufacturer's information. Plasma was applied for 90 s while the electrode's surface ensured a constant gap of 2 mm filled with ambient air.

Microwave-driven pulsed plasma jet (CAP III), described in detail by Lehmann et al.,[1] was generated by means of the following parameters: He flow 2.0 slm, N_2 flow 0.3 slm, pulse power 200 W, pulse width 5 µs, pulse repetition frequency 4.9 kHz, resulting in an average input power of 5 W. For a meandering movement a three-axis linear motion system (Steinmeyer MC-G047) was used, with a scanning speed of 8 mm/s, line spacing of 0.1 mm, and working distance of 3 mm from the sample surface; the plasma jet was applied for 90 s on each specimen.

For the mechano-antimicrobial procedure (positive control, $n = 8$), specimens were mechanically cleaned with a plastic curette (Hu-Friedy, Frankfurt am Main, Germany) for 10 s and placed in 2 mL of 0.2% chlorhexidine (CHX) for 90 s (Chlorhexamed forte, GSK, Berlin, Germany).

2. Air–Water Spraying

After treatment with CAP or mechano-antimicrobial procedure or no treatment (negative control, $n = 8$) specimens were air–water sprayed for 10 s using a dental air/water spray unit (U 3000 EX, Ultradent, Brunnthal, Germany). The input water pressure was set to 2 bar.

C. Microbiology

All titanium discs ($n = 40$) were inoculated for 5 s twice on brain heart infusion blood agar (Sigma-Aldrich, Taufkirchen, Germany) on Rodac plates (d: 50mm, Merck, Darmstadt). Eight further fresh specimens without biofilm were used as controls. After inoculation, agar plates were incubated at 37°C (5% CO_2). The plates were examined after 24 h and 48 h of incubation.

D. Fluorescence Microscopy

The existence and amounts of biofilms on the titanium discs were assessed by fluorescence microscopy using live/dead staining (Live/Dead BacLight Bacterial Viability Kit L7012, Molecular Probes, Carlsbad, CA, USA). Eight more specimens without biofilms were used as controls. The live/dead stain was prepared by diluting 1 µL of SYTO 9 and 1 µL of propidium iodide in 1 mL of NaCl solution and stored for 60 min on ice cubes in a dark container. The titanium specimens were placed in 24-well plates and 10 µL of the reagent mixture was added to each

well, followed by incubation at room temperature in the dark for 15 min. Specimens were placed carefully on a glass slide and covered with mounting oil and a glass cover slide.

The biofilm analysis was performed using a reverse light fluorescence microscope (Leitz DMR, Leica, Wetzlar, Germany) provided with a digital camera (AxioCam MRm Rev. 3, Carl Zeiss Microimaging, Göttingen, Germany) and corresponding filter sets using the image processing software ZEN 2.3 (Carl Zeiss Microimaging).

The biofilm coverage of the specimens was captured by taking images of the titanium surface in two randomly chosen areas of each titanium sample. Red and green color images were taken separately. Medians of red and green fluorescence intensities were calculated using the 0–255 gray scale and total fluorescence and percentage of areas without fluorescence were ascertained by red/green overlays. Continuous data with median and interquartile ranges were summarized (25–75th percentile).

E. Scanning Electron Microscopy (SEM)

The biofilm's quality was analyzed by SEM on one randomly selected specimen of each subgroup. A titanium disc without biofilm served as a control. The specimens were fixed in glutaraldehyde (2.5% in phosphate-buffered saline [PBS]; PAA laboratories GmbH, Pasching, Austria) for 50 min, followed by rinsing four times for 10 min in PBS. Subsequently, the specimens were dehydrated using an increasing series of ethanol rinses (50–90% 10 min each; 96% 2 × 10min). Last, the samples were dried in 1,1,1,3,3,3-hexamethyl-disilazane (HMDS, Acros Organics, Geel, Belgium). HMDS was vaporized in a clean bench at room temperature. All specimens were mounted on SEM sample stubs (Plano, Wetzlar, Germany), followed by platinum sputtering. SEM analysis was performed in an FEI XL30 ESEM FEG (FEI Company, Eindhoven, The Netherlands) at magnifications of 1000× to 10,000×. Each subgroup's titanium surface was scanned for biofilm remnants or bacteria as well as structural changes caused by plasma treatments in triplicates.

F. Statistical Analysis

Statistical analysis was performed using SPSS 24 (IBM, Armonk, IL, USA).

III. RESULTS

All test groups showed a lowered growth on Rodac plates (Figs. 1 and 2) compared with the control group. The fluorescence microscopy analysis (Fig. 3) showed significantly lowered biofilm coverage in all test groups compared with the control group (*t* test, $p < 0.05$) (Figs. 4 and 5). Within the test groups, the only significant difference was between CAP II and CAP III ($p = 0.032$). SEM confirmed these results, showing disintegrated biofilms in all test groups (Table 1).

FIG. 1: Microwave-driven pulsed plasma, IOM source (CAP III), on titanium discs

IV. DISCUSSION

To the best of our knowledge, this is the first study to compare three different CAP sources in terms of disintegrating multispecies *in vivo*-formed oral biofilm on infected titanium surfaces. It was hypothesized that exposure of CAP leads to significant lower biofilm coverage defined by more effective disintegration and inactivation of e*x vivo* multispecies biofilms.

It was demonstrated that all three CAPs, indirect plasma kINPen® MED, direct plasma PlasmaDerm FLEX 9060, and microwave-driven pulsed indirect plasma, led to significant reduction in vitality and coverage of 72 h *in vivo*-developed biofilm on titanium discs (*t* test, $p < 0.05$). Furthermore, it was found that the effectiveness of disintegrating biofilms with all applied plasma sources was comparable to mechanical debridement combined with 0.2% CHX treatment. The experimental microwave plasma source[34] reduced the biofilms significantly more than the direct plasma source ($p = 0.032$). None of the other groups was significantly different in biofilm removal. Our hypothesis could therefore only be partially confirmed. In a former similar *ex vivo* study,[32] it was possible to totally remove biofilms on titanium specimens. These results differ from those in our present study. In contrast to our current procedure, both the CAP and the air–water spray were used twice. This resulted in a considerable additional expenditure of time. However, because the procedure in our current study also led to a considerable biofilm reduction and biofilm disinfection,

FIG. 2: Rodac plates results coverage. (a) CAP I kINPen® MED. (b) CAP II PlasmaDerm FLEX 9060. (c) CAP III IOM source. (d) Positive control CHX. (e) Negative control.

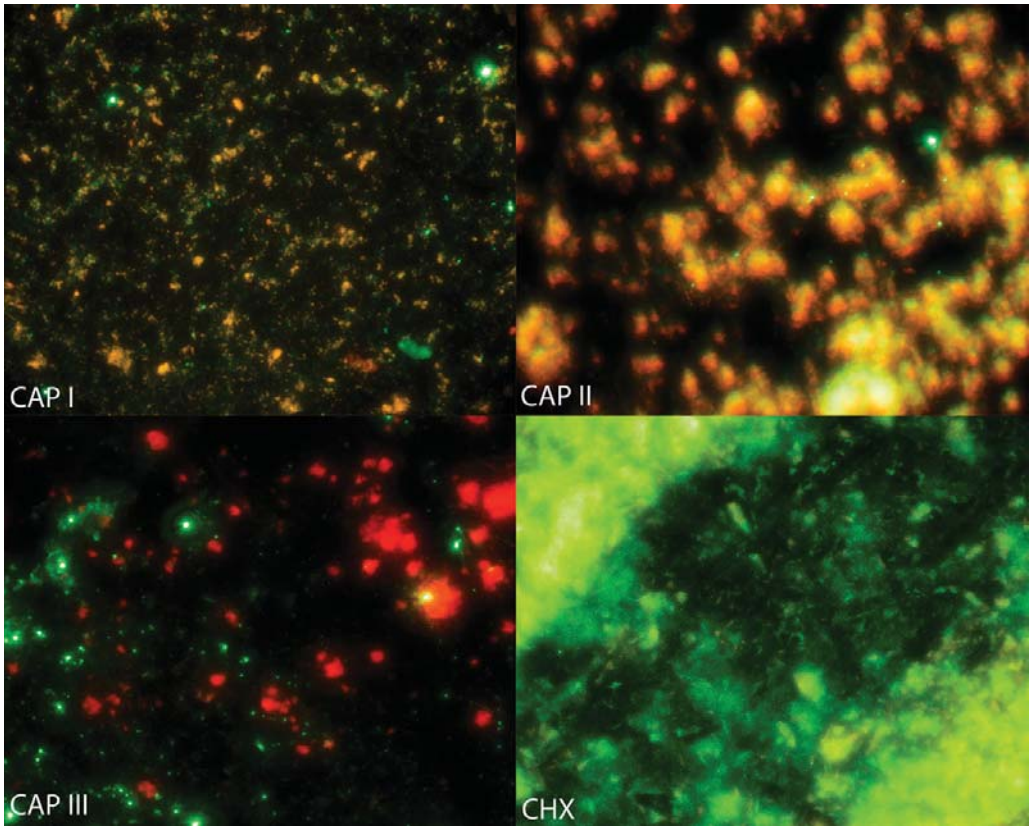

FIG. 3: Fluorescence microscopy results biofilm coverage. (a) CAP I kINPen® MED. (b) CAP II PlasmaDerm FLEX 9060. (c) CAP III IOM source. (d) Positive control CHX.

parameters are available that can be adapted to the corresponding clinical requirements of biofilm removal or biofilm disinfection.

Another study demonstrated that indirect plasma (kINPen® MED) removed artificial biofilm *in vitro* more effectively than application of a diode laser[35] and *in vivo*-developed biofilm on extracted teeth could been removed in the analog dimension as by mechanical cleaning.[36] In another *in vitro* study, a CAP application of 600 s to *Pseudomonas aeruginosa* monospecies biofilms could achieve higher antimicrobial reduction than treatment with 0.1% CHX.[37] The results of these two studies also support our conclusion that CAP should not be used exclusively, but rather adjunctively.

To optimize the material properties of titanium surfaces, studies could demonstrate an improved wettability as well as optimized growth behavior of fibroblasts and osteoblasts after plasma treatment.[38,39] Additionally, an optimized osseointegration of implants after 6 weeks was found after treatment with CAP in a dog

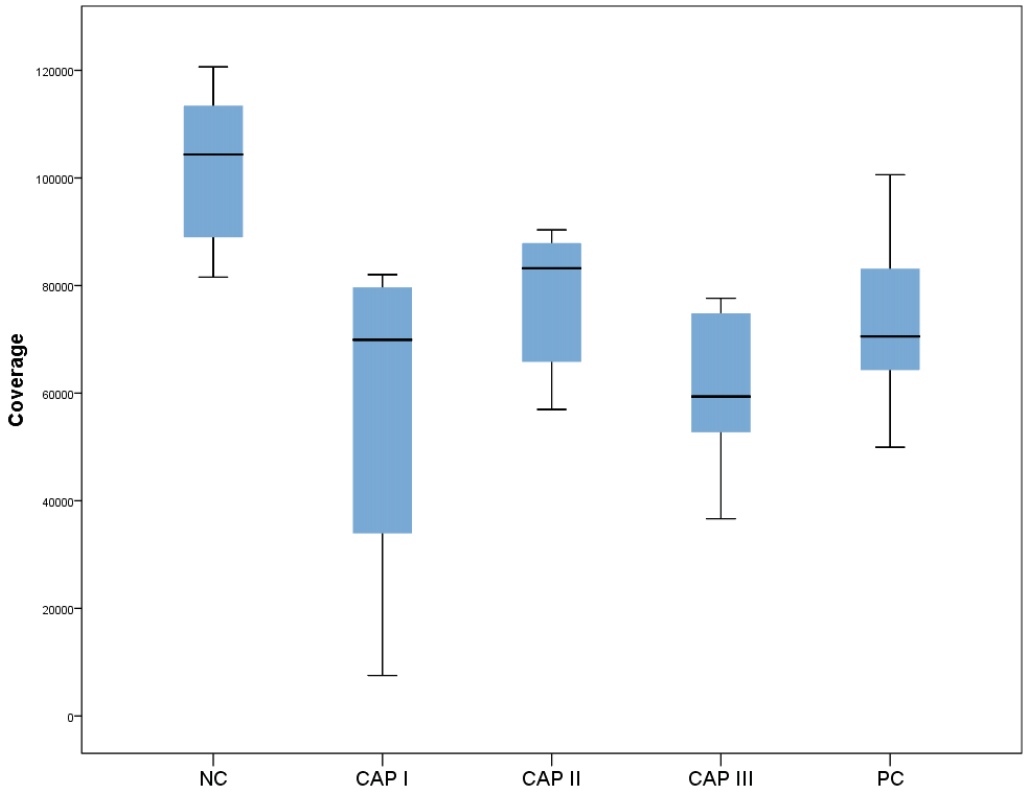

FIG. 4: Box plots results of biofilm coverage

model.[40] Beyond the regeneration of bone structures, plasma could have the potential to accelerate wound healing of peri-implant inflammations.[7,41] Until now, no resistances of specific microbes or permanent unwanted effects are known for CAP treatment.[7,13,14,42–44]

In summary, the results of the present study demonstrate the potential of different CAP sources to reduce oral multispecies biofilms and to disinfect titanium surfaces for use in regenerative peri-implantitis therapy. In conjunction with the results of other studies, we assume that adjuvant application of CAP in combination with mechanical debridement and antimicrobial agencies leads to increased antimicrobial effectiveness and eliminates biofilm more effectively than mono-therapies.[32,35,38] There is no method currently in existence that completely disinfects and eliminates biofilm on implant surfaces without destroying the material properties of the surface. Plasma treatment could represent a non-invasive, time-efficient therapy option. Further studies are necessary to investigate how adjunctive plasma biofilm treatment could be optimized.

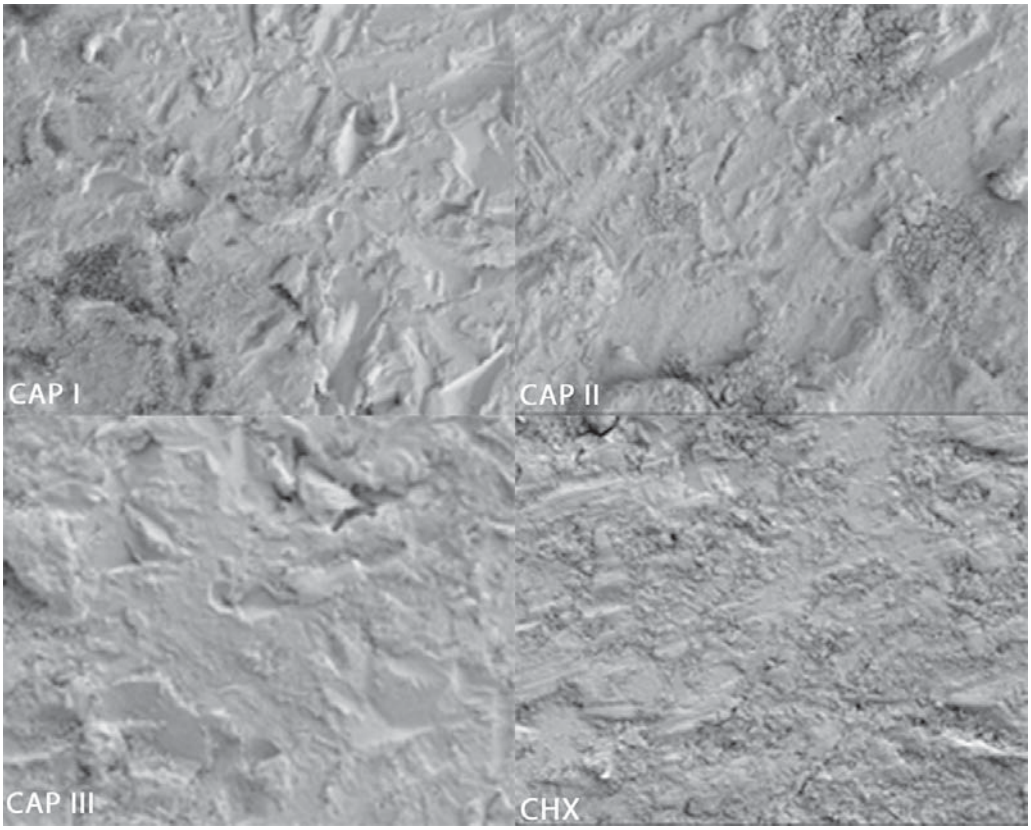

FIG. 5: SEM results of biofilm coverage. Magnification, 2000×, HV 1.0 kV, Spot 3.0, 50.0 µm. (a) SEM CAP I kINPen® MED. (b) SEM CAP II PlasmaDerm FLEX 9060. (c) SEM CAP III IOM source. (d) SEM positive control CHX.

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TABLE 1: Results of culture, fluorescence microscopy, and SEM

	Culture colonies		Fluorescence microscopy Median coverage (%)	SEM disintegrated biofilm Yes/no
	Complete	Few		
CAP I	—	X	72.77	Yes
CAP II	—	X	86.83	Yes
CAP III	—	X	69.49	Yes
CHX	—	X	70.40	Yes
NC	X	—	100.00	No

NC, negative control

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