Chlorin e6 mediated photodynamic inactivation for multidrug resistant Pseudomonas aeruginosa keratitis in mice in vivo

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Following corneal epithelium scratches, mouse corneas were infected with the multidrug resistant (MDR) P. aeruginosa strain PA54. 24 hours later, 0% (for control group), 0.01%, 0.05% or 0.1% Chlorin e6 (Ce6), a second generation photosensitizer derived from chlorophyll, was combined with red light, for photodynamic inactivation (PDI). 1 hour or 2 days later, entire mouse eyes were enucleated and homogenized for counting colony forming units (CFU) of P. aeruginosa. For comparison, 0.1% Ce6 mediated PDI was started at 12 hours post infection, and 0.005% methylene blue mediated PDI 24 hours post infection. Clinical scores of corneal manifestation were recorded daily. Compared to the control, CFU 1 hour after PDI started 24 hours post infection in the 0.01% Ce6 and 0.05% Ce6 groups were significantly lower (more than one log₁₀ reduction), the CFU 2 days post PDI higher in the 0.1% Ce6 group, clinical score lower in the 0.1% Ce6 group at 1 day post PDI. These findings suggest that PDI with Ce6 and red light has a transient efficacy in killing MDR-PA in vivo, and repetitive PDI treatments are required to fully resolve the infection. Before its clinical application, the paradoxical bacterial regrowth post PDI has to be further studied.

Pseudomonas aeruginosa (P. aeruginosa), a Gram-negative rod-shaped bacterium, is among the most common pathogens of bacterial keratitis. P. aeruginosa keratitis usually causes a rapidly progressive, suppurative corneal infiltration, ulceration, and finally perforation and blindness. Infections caused by P. aeruginosa are further complicated by the fact that multidrug resistant (MDR) variants of this species are on the rise worldwide, which are difficult to treat by conventional therapy, the empirical but intensive topical treatment with commonly used ophthalmic antibiotics, such as the fluoroquinolones ciprofloxacin and ofloxacin, the aminoglycosides gentamicin and tobramycin, and the 1st generation cephalosporin cefazolin, respectively. Many risk factors, such as contact lens use, compromised ocular surface and preservative-free lubricant ointment use, can predispose patients to P. aeruginosa keratitis. P. aeruginosa may also cause keratitis after keratoplasty, which often leads to a very poor outcome. The development of alternative therapy options to fight this type of infection has become imperative.

Photodynamic inactivation (PDI) is a novel promising antimicrobial strategy against MDR bacteria. It is a photochemical reaction combining light, a photosensitizer, and oxygen to generate reactive oxygen species (ROS), such as singlet oxygen (¹O₂) and hydroxyl radicals (HO). Unlike antibiotics, PDI causes a non-specific bacterialic effect, since ROS can damage various vital bacterial structures, mainly cell wall proteins and membranes.

PDI might be especially applicable for the treatment of infectious keratitis, since the transparency and superficial location of the cornea facilitate the deposition of the photosensitizer and the irradiation process. As early...
as in the 1970s, some clinical studies already tried to treat viral keratitis with PDI, but these investigations were soon suspended due to severe adverse reactions, such as phototoxic epithelial keratitis and anterior uveitis. In the following decades, only in vitro studies that explored the possibility of PDI to combat clinically important corneal pathogens were reported. To date, the in vivo use of PDI as antimicrobial therapy is mainly restricted to the treatment of dermatological and dental infections. In ophthalmology, PDI was rarely used as a treatment option for ocular infections, although another photodynamic therapy procedure, i.e. corneal crosslinking with riboflavin and ultraviolet light, is nowadays commonly applied as a treatment for corneal ectatic diseases. A small number of clinical case reports indeed suggest that corneal crosslinking might be used as a therapy option for bacterial keratitis, while other case reports indicated an increase in microbial infections as a complication of this therapy form when treating corneal ectatic diseases. To date, only little experimental evidence is provided, whether PDI is a useful option to fight bacterial keratitis. In 2011, Shih et al. demonstrated the bactericidal efficiency of a combination of the antibiotic amikacin and PDI with the photosensitizer methylene blue (MB) and red light (λ = 650 nm) in a rabbit model of Mycobacterium fortuitum keratitis. However, they did not detect a statistically significant impact of this therapy regimen.

In the present study, we evaluated the efficiency of PDI with the photosensitizer Ce6 and red light (λ = 670 nm) as a single therapy option for the treatment of MDR P. aeruginosa keratitis in mice. Previous in vitro studies demonstrated that this photosensitizer/light combination can kill up to 100% of the challenged P. aeruginosa cells. However, whether such a remarkable bactericidal rate can be achieved in vivo is questionable. Under in vitro conditions, bacteria can be fully mixed with the photosensitizer, and the generated ROS will predominantly damage the bacterial cell components. The in vivo environment is much more complicated. Corneal epithelium and stroma may hinder the diffusion of the photosensitizer towards the bacteria within the infected tissue. ROS can diffuse for only short distance, e.g. less than 50 nm, once they are generated from the activated photosensitizer. Thus, a substantial proportion of ROS produced by PDI is likely to react with host components, including cellular structures and molecules, and may cause unexpected cascade reactions.

To assess the efficiency of PDI in vivo, three regimens of PDI were tested (Fig. 1). In all regimen, visible red light (λ = 670 nm) was applied, thereby avoiding the potential genotoxicity induced by ultraviolet light used in corneal crosslinking.

In regimen I, at 24 h post MDR-PA infection, corneal epithelia were removed from infected mouse eyes using a hockey epithelium removal knife to improve the diffusion of the photosensitizer into the stroma, before PDI mediated by 0%, 0.01%, 0.05%, and 0.1% Ce6 and red light was applied. Mice were euthanized either 1 h post PDI or 48 h post PDI, the infected eyes enucleated and subsequently homogenized with a manual disperser for CFU determinations as outlined in Material and Methods.

In regimen II, PDI mediated by 0% and 0.1% Ce6 was carried out 12 h post infection on deep epithelialized corneas, and mice were euthanized 60 h post PDI for CFU counting. Inflammatory cells, such as neutrophils, which are recruited to the cornea due to the P. aeruginosa infection, are essential for controlling the bacterial population. However, an excessive influx and persistence of these inflammatory cells may also lead to corneal destruction. PDI may interfere with the corneal response to P. aeruginosa unintentionally by killing these inflammatory cells. Of note, Tanaka et al. reported that a MB concentration of around 100 μM, when activated with a red light dose of 20 J/cm², spared neutrophils but killed most of the bacteria under in vitro conditions.

Therefore, in regimen III, we used MB as a reference to investigate whether it causes a similar effect as Ce6. PDI mediated by 0.005% MB was carried out at 24 h post infection. Mice were euthanized 48 h post PDI for CFU counting.
Results

Regimen I (Ce6 mediated PDI at 24 h post infection). In the first regimen, PA54 infected eyes were treated 24 h post infection by PDI with different concentrations of Ce6 and red light, and bacterial loads were subsequently determined in homogenates of whole eyes enucleated 1 h and 48 h post PDI treatment, respectively, in order to determine the short term and long term effects of PDI on the bacterial loads in the infected eyes (Fig. 2). The CFU values found in the eyes of PA54 infected mice 1 h after PDI are shown in Fig. 2A. Upon PDI the bacterial loads found in mice treated with the 0.01% Ce6 gel ($\log_{10}$CFU = 5.19 ± 0.75, $P = 0.028$) and the 0.05% Ce6 gel ($\log_{10}$CFU = 5.13 ± 0.61, $P = 0.015$) decreased significantly when compared to the CFU rates found in the eyes of control mice (0.1% Ce6), however, this difference was not statistically significant ($P = 0.058$).

A different picture emerged when the bacterial loads of PA54 infected mice were determined 48 h post PDI (Fig. 2B). Here, the bacterial loads in the eyes of mice treated with the 0.1% Ce6 gel ($\log_{10}$CFU = 7.38 ± 0.22) increased significantly when compared with the CFU rates found in the eyes of the sham treated control group ($\log_{10}$CFU = 6.95 ± 0.25, $P = 0.011$). Likewise, CFU rates found in the eyes of the 0.05% Ce6 gel treated group ($\log_{10}$CFU = 7.19 ± 0.13), and the 0.01% Ce6 gel treated group ($\log_{10}$CFU = 7.11 ± 0.27) exceeded the CFU values observed in the eyes of the sham-treated control group, however, this was not statistically significant ($P = 0.068$ and 0.333, respectively).

The course of infection in the eyes of mice challenged with PA54 was additionally monitored by stereo-microscopic determinations and graded as outlined in Materials and Methods. The clinical scores from PA54 infected eyes determined 24 h, 48 h, and 72 h post infection, respectively, are shown in Fig. 3, and representative stereo-microscopic images of PA54 infected eyes over time are given in Fig. 4. Here, significant increases in infection severity were observed for the sham treated group and the 0.01% Ce6 gel, when the development of infection symptoms over time within a treatment group were compared. The clinical scores of 0.01% Ce6 gel...
Our current study assessed the effectiveness of corneal PDI by the photosensitizer Ce6 and red light (λ = 670 nm) against MDR-PA keratitis in a murine infection model. We observed that a single PDI treatment with this photosensitizer/light combination significantly decreased the bacterial load in infected eyes by more than 90% at one h
An interesting finding is that the highest killing efficiency of PDI by Ce6/red light was not achieved by the highest concentration of Ce6, i.e. 0.1%, but rather by lower Ce6 concentrations. Such a nonlinear correlation between concentration and photodynamic inactivation effect has been observed for many photosensitizers, and is in line with our previous in vitro findings, indicating a dose-dependent bactericidal effect of PDI by Ce6 and red light against clinical P. aeruginosa isolates. An explanation for this finding might be that an excess of the photosensitizer decreases the transmission of light required for efficient singlet oxygen generation. Alternatively, higher Ce6 concentrations might induce the expression of export systems in P. aeruginosa resulting in a decreased concentration of the photosensitizer at/in the bacterial cell. However, the molecular mechanisms responsible for

Figure 4. Representative corneal manifestations of mice treated by regimen I (chlorin e6 mediated PDI at 24 h post infection), in which PDI was mediated by (A,B,C) 0%, (D,E,F) 0.01%, (G,H,I) 0.05% and (J,K,L) 0.1% chlorin e6 (Ce6). Photos were taken (A,D,G,J) right before infection of P. aeruginosa (hour 0), (B,E,H,K) before PDI (hour 24), and (C,F,I,L) on day 3. (E) A characteristic infiltration ring of P. aeruginosa keratitis (clinical score 3). The corneal suppurative infiltration developed rapidly in all mice, but on day 3, it seemed to be milder in (L) the mouse treated by 0.1% Ce6 mediated PDI (clinical score 3) than the others (clinical score 4).
decreased susceptibility of *P. aeruginosa* cells against PDI in presence of increased concentrations of Ce6 still need to be clarified.

The observations of equal to higher CFU values in the eyes of Ce6 treated mice at 72 h post infection (i.e. 48 h post PDI treatment) demonstrated on the other hand that a single PDI treatment was insufficient in resolving the infection *in vivo*. Especially the significant bacterial overgrowth induced by the 0.1% Ce6 PDI treatment at day 3 of regimen I is a matter of concern, although this bacterial overgrowth was not seen when Ce6 concentrations ≤0.05% were utilized. This finding is supported by previous clinical case reports, indicating an increased risk of microbial infections as a complication that may be induced by corneal crosslinking29–38.

A solution to the possible bacterial overgrowth is crucial for the long-term safety of corneal PDI. A safe corneal PDI can be applied repeatedly on the infected cornea until all bacteria are eradicated, which certainly maximizes the potency. To explore a safer PDI protocol, we carried out the experiments of regimen II and regimen III. Since *P. aeruginosa* overgrowth, the main problem, was on day 3, we only measured the CFU on day 3 in regimen II and regimen III.

In regimen II, we focused on 0.1% Ce6, as only this photosensitizer concentration induced a significant bacterial overgrowth in regimen I. When animals were treated once by PDI with 0.1% Ce6 at 12 h post infection, the clinical scores on day 3 did no longer differ from those seen in the eyes of the sham treated control group, suggesting that the risk of bacterial overgrowth might be decreased when PDI can be applied early in infection.

An inconsistency between the milder corneal symptom and higher bacterial load was found in the group treated by 0.1% Ce6 mediated PDI on day 3 of regimen I. A possible explanation might be that a higher proportion of inflammatory cells (mainly neutrophils) recruited to the focus of corneal infection were killed by this PDI regimen, leading to a reduced inflammatory response at the later stage of infection, however, at the cost of bacterial overgrowth.

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**Figure 5.** Log$_{10}$CFU on day 3 of each mouse treated by regimen II (chlorin e6 mediated PDI at hour 12 post infection) is displayed as a single dot in the scatter plot. Mean of Log$_{10}$CFU of each group is shown as a bar. 0.1% chlorin e6 (Ce6) was used in PDI at hour 12, while 0% Ce6 was used in control group. There was no significant difference between the two groups.

**Figure 6.** Clinical scores of mice treated by regimen II (chlorin e6 mediated PDI at hour 12 post infection), recorded from hour 12 to day 3. Means with standard deviations are shown. 0.1% chlorin e6 (Ce6) was used in PDI at hour 12, while 0% Ce6 was used in control group. There was no significant difference between the two groups. Clinical scores of 0% Ce6 group increased significantly from hour 12 to day 2 (*P* = 0.002), and from hour 12 to day 3 (*P* = 0.002). Similarly, clinical scores of 0.1% Ce6 group increased significantly from hour 12 to day 2 (*P* = 0.002), and from hour 12 to day 3 (*P* = 0.002).
To test this hypothesis, we used the photosensitizer MB in combination with red light for PDI, which was reported to spare neutrophils at concentrations of about 100 μM, and compared its effectiveness to kill *P. aeruginosa* in the murine corneal scratch model with that of Ce6 mediated PDI. Taking into account the insufficient diffusion of this photosensitizer into the corneal stroma, the molar concentration of 0.005% MB used in regimen III was 156 μM, and thus slightly higher than the 100 μM MB recommended in a previous *in vitro* study, but just equivalent to 0.009% Ce6. Moreover, for the same amount of substance, the ROS generated from irradiated MB is just about 80% of that of Ce6. Clinical scores of the group treated by MB mediated PDI were not different from those of the control group, suggesting that the corneal infiltration was not markedly affected by this PDI treatment. Yet in regimen III, the group treated by MB mediated PDI showed a considerable tendency of bacterial overgrowth on day 3, compared with the control group (*P* = 0.060), while the group treated by 0.01% Ce6 mediated PDI did not show such a bacterial overgrowth on day 3 (*P* = 0.333). This finding is supported by the previous...
study that shows higher efficiency of Ce6 in comparison with MB in PDI of Gram-positive bacteria in vitro\(^5\). Since MB is not better than Ce6 in preventing bacterial overgrowth either, further study is required to compare more different photosensitizers.

Considering the potential of PDI as treatment option against infectious keratitis induced by MDR bacteria, additional experiments are warranted to better uncover the mechanism(s) that promote the bacterial regrowth after PDI treatment. The overall susceptibility of a pathogen to PDI may also have a profound effect on the bacterial regrowth after PDI. PDI of Gram-negative bacteria is more challenging than of Gram-positive bacteria, because Gram-negative bacteria are surrounded by a less permeable envelope consisting of an inner cytoplasmic membrane and an outer membrane, while Gram-positive bacteria are surrounded by a single cytoplasmic membrane and a porous cell wall\(^5\). The overall outer membrane permeability of \(P.\ aeruginosa\) is even lower than that of most other Gram-negative bacteria, for example, 12 to 100-fold lower than that of \(E.\ coli\)\(^6\). Thus, PDI might be particularly suited as therapy option against keratitis caused by MDR Gram-positive bacteria.

After all, Ce6, and additionally MB, are just two of the most basic photosensitizers applied for PDI. A large number of novel photosensitizers have been developed recently. In some in vitro studies, it has been suggested that polycationic conjugates of poly-L-lysine and Ce6 have higher efficiency for Gram-negative bacteria than free Ce6\(^42,55\). Some photosensitizers even allow selective targeting and killing bacteria in the presence of mammal cells\(^56-58\). The development of the latter group of photosensitizers can not only improve the antibacterial killing potency, but also very likely to affect the safety of PDI. We are looking forward to further studies that address the in vivo efficiency of these novel substances against bacterial corneal infections.

In comparison with PDI, many other studies looked at different experimental approaches for treating \(P.\ aeruginosa\) keratitis. An animal study has compared the efficacy of antibiotics to treat \(P.\ aeruginosa\) keratitis\(^59\), and found that moxifloxacin, levofloxacin and ciprofloxacin were equally effective against the \(P.\ aeruginosa\) isolate. However, when keratitis is caused by a fluoroquinolone-resistant \(P.\ aeruginosa\) isolate, which are on the rise in many regions of the world, this class of antibiotics won't be any longer a valid therapy option. Some other novel tools, such as bacteriophages and antimicrobial peptides (AMPs), are now under development, and each tool has its own risks and advantages. A recent study indicated for example the efficacy of bacteriophage KPP12 for the treatment of \(P.\ aeruginosa\) keratitis in vivo\(^60\). However, this study demonstrated that the bacteriophage failed to lyse about one third of the clinical \(P.\ aeruginosa\) strains tested, indicating that this therapy is likely to fail in a large number of cases. The AMP esculentin-1a(1–21)NH₂, a frog skin-derived peptide, was also recently reported to significantly reduce the severity of infection in a murine \(P.\ aeruginosa\) keratitis model\(^61\), a three \(log_{10}\) reduction was achieved when the AMP was applied three times per day for four days post infection. However, the authors noted that the disease was still progressive even under this intensive regimen.

In summary, our current study has proven that Ce6 mediated PDI is able to cause about one \(log_{10}\) reduction of the MDR-PA cell pool in a murine keratitis model. However, the high numbers of bacterial cells seen at later stages post PDI suggest that consecutive rounds of PDI are required to reduce the amount of bacterial cells in infected eyes in a way that the host immune system can control the infection. The differences in bacterial growth observed after PDI with increasing photosensitizer concentration indicate furthermore that care needs to be taken to identify the optimal dose of photosensitizer to effectively decrease the bacterial burden without causing too many side effects that may interfere with bacterial clearance.

Materials and Methods

Materials. Agar plate of Trypticase\(^\text{TM}\) soy agar with 5% sheep blood (TSBA) (Becton Dickinson GmbH, Heidelberg, Germany). Difco\(^\text{TM}\) LB broth, Lennox (LB-L) (Becton, Dickinson and Company, Sparks, USA). Midazolam (Hameln Pharma Plus GmbH, Hameln, Germany). Fentanyl (Hameln Pharma Plus GmbH, Hameln, Germany). Domitor\(^\text{TM}\) medetomidine hydrochloride (Orion Corporation, Espoo, Finland). Naloxone hydrochloride (Inresa Arzneimittel GmbH, Freiburg, Germany). Flumazenil (Inresa Arzneimittel GmbH, Freiburg, Germany). Antisedan\(^\text{TM}\) atipamezole hydrochloride (Orion Corporation, Espoo, Finland). Sodium chloride (VWR International Ltd., Leuven, Belgium). Chlorin e6 gel (using hydroxypropyl methylcellulose as gel-base)
Bacterial strain and culture conditions. The MDR *P. aeruginosa* strain PA54, isolated from human infection, was collected in 2009 at the Institute of Medical Microbiology and Hygiene, Saarland University Medical Center, Homburg, Germany. The resistance profile of PA54 was determined using the automated VITEK 2 system (bioMérieux GmbH, Nürtingen, Germany). PA54 was routinely cultivated overnight on TSBA at 37°C.

Murine corneal scratch model. Mice. In preliminary experiments, we found that in mice younger than 6 months, *P. aeruginosa* keratitis showed a trend of spontaneous resolution during one week. Therefore, we used eight-to-eleven month old female C57BL/6N mice, which were purchased from Janvier (Le Genest Saint Isle, France) and kept under specific pathogen-free conditions according to the regulations of German veterinary law. All experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, USA), and were approved by the local State Review Boards (Landesamt für Soziales, Gesundheit und Verbraucherschutz) of Saarland following the national guidelines for the ethical and humane treatment of animals.

Inoculum preparation. PA54 was grown overnight in LB-L at 37°C, diluted in fresh medium in a ratio of 1:100, and incubated at 37°C and 150 rpm shaking for 3 hours. Bacteria from the exponential growth phase were harvested by centrifugation, washed twice in PBS, resuspended in fresh LB-L to an optical density at 600 nm (OD600) of 10. The number of viable cells was determined by plating out serial dilutions on TSBA. CFU were counted after incubation overnight at 37°C.

Infection. The time of infection of mice was deemed as hour 0 on day 0 of the experiment. Mice were narcotized by intraperitoneal injection of 10 μl/g anesthetic consisting of 5 μg/ml Fentanyl, 0.5 mg/ml midazolam and 0.05 mg/ml medetomidine hydrochloride in 0.9% sodium chloride solution. The natural tear flow was suppressed by dropping 10 μl of a 0.6% acetyl-cysteine solution onto the cornea. Subsequently, mice were placed under a stereo-microscope, and three linear scratches (each ~2 mm) were made on the ocular surface of the left eye of each mouse by a 27 gauge syringe needle to expose the subepithelial stroma. 5 μl of the PA54 suspension, corresponding to 1 × 10⁶ CFU, were pipetted onto the mechanically harmed cornea when mice were lateral recumbent. The bacterial solution was left on the eye for 20 minutes, and mice were subsequently awakened by injecting 10 μl/g antidote consisting of 0.12 mg/ml naloxone hydrochloride, 0.05 mg/ml flumazenil and 0.25 mg/ml atipamezole hydrochloride in 0.9% sodium chloride solution.
Grading of corneal infections. The corneal manifestation of the PA54 induced keratitis in the infected mouse eyes were examined daily under a stereomicroscope, and recorded as a clinical score using the following scale: Score 0, entire cornea clear; score 1, <50% cornea is semi-transparent (iris color behind the lesion can be identified); score 2, >50% cornea is semi-transparent; score 3, <50% cornea is opaque (iris color behind the lesion cannot be identified); score 4, >50% cornea is opaque; score 5, corneal spontaneous perforation or phthisis bulb. Corneal photos were taken under the stereomicroscope when mice were in anesthesia.

Bacterial load determinations. Enucleated mouse eyes were homogenized with a POLYTRON PT 1200 E Manual Disperser (Kinematica AG, Lucerne, Switzerland) in 1 ml LB-L, and serial dilutions of this homogenate plated on TSBA. After 24 h of incubation at 37 °C, CFU were counted.

PDI studies. All steps involving Ce6 were carried out in a dark room under weak yellow light exposure (λ = 596 nm) to avoid activation of the photosensitizer. Three regimens of PDI were tested (Fig. 1). In all regimens, corneal epithelia of narcotized mice were removed by a hockey epithelium removal knife prior to PDI. The irradiation setting to activate Ce6 was identical in all regimens, with 6 minutes irradiation of red light (λ = 670 nm), equivalent to an energy dose of 24 J/cm².

Regimen I. At 24 h post infection, corneal epithelia were removed from infected eyes, and about 50 µl of a 0.01% (hydroxypropyl methylcellulose gel-base only), 0.05%, and 0.1% Ce6 gel dabbed onto the infected mouse cornea, respectively. Mice were then left in darkness for 30 minutes to allow the penetration of Ce6 into the corneal tissues. Afterwards, gel residues were carefully removed with cotton swabs, and mouse corneas irradiated as indicated above. Mice were euthanized either 1 h post PDI or 48 h post PDI, the infected eyes enucleated and subsequently homogenized for CFU determinations.

Regimen II. Corneal epithelia were removed from infected eyes at 12 h post infection, and about 50 µl of a 0.005% MB solution and PBS dropped onto the infected mouse cornea, respectively. The PDI treatment was carried out as described before. Mice were euthanized 60 h post PDI and handled as described above.

Regimen III. Corneal epithelia were removed from infected eyes at 24 h post infection, and about 20 µl of a 0.005% MB solution and PBS dropped onto the infected mouse cornea, respectively. The PDI treatment was carried out as described before. Mice were euthanized 48 h post PDI, and handled as described above.

Statistical analysis. Statistical analysis was performed with IBM SPSS Statistics 22.0. Since bacterial growth data is a typical variable that follows the log-normal distribution62, after log transformation of the CFU values, independent samples t test following analysis of variance was used to compare Log10CFU. Mann-Whitney U test -normal distribution62, after data is a typical variable that follows the log transform of the CFU values. P < 0.05 was considered statistically significant.
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**Author Contributions**
M.B. and T.T. designed the experiments. M.B., T.T., D.H. and H.J.F. provided experimental devices and materials. M.F.W. and M.D. performed the experiments. M.L. analyzed the data. M.F.W. wrote the main manuscript text. M.F.W. and N.S. prepared figures. M.B. and B.S. proofread the manuscript. All authors reviewed the manuscript.

**Additional Information**

**Competing Interests:** The authors declare no competing financial interests.

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