Photomechanical Drug Delivery into Bacterial Biofilms

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Purpose. To investigate whether photomechanical waves generated by lasers can increase the permeability of a biofilm of the oral pathogen *Actinomyces viscosus*.

Methods. Biofilms of *Actinomyces viscosus* were formed on bovine enamel surfaces. The photomechanical wave was generated by ablation of a target with a Q-switched ruby laser and launched into the biofilm in the presence of 50 μg/ml methylene blue. The penetration depth of methylene blue was measured by confocal scanning laser microscopy. Also, the exposed biofilms were irradiated with light at 666 nm. After illumination, adherent bacteria were scraped and spread over the surfaces of blood agar plates. Survival fractions were calculated by counting bacterial colonies.

Results. Confocal scanning laser microscopy revealed that a single photomechanical wave was sufficient to induce a 75% increase in the penetration depth of methylene blue into the biofilm. This significantly increased the concentration of methylene blue in the biofilm enabling its photodestruction.

Conclusions. Photomechanical waves provide a potentially powerful tool for drug delivery that might be utilized for treatment of microbial infections.

KEY WORDS: photomechanical wave; biofilm; *A. viscosus*; methylene blue; confocal scanning laser microscopy.

INTRODUCTION

Most microbial infections in the body are caused by bacteria growing as a mono- or a multi-species biofilm (1). Biofilms are matrix-enclosed microbial aggregates associated with each other and a solid surface (2). Bacteria within biofilms have an increased (up to 1500-fold higher) resistance to antimicrobial agents compared with that of planktonic cells of the same species (3). This may be due to structural (4) and metabolic factors (5) as well as the development of biofilm/attachment-specific phenotypes (6,7).

Ultrasound (8) and electric fields (9,10) have been used to enhance the efficacy of antibiotics in killing biofilm bacteria in sterilization processes. Ultrasonic irradiation enhanced the killing of *Pseudomonas aeruginosa* in biofilms by gentamycin by nearly two orders of magnitude (8). Small direct current electric fields achieved a 6- to 8-log increase in killing after 24 h of exposure to the direct current using biofilms of *P. aeruginosa* (9,10). On the other hand, photomechanical waves (PW) generated by ablation with high pulse lasers are wideband (fast risetime) compressional waves which do not have a measurable tensile component (11). While the action of ultrasound is primarily mediated by heat and cavitation (12), PW effects are caused by mechanical forces (13). PW have been shown to induce a transient increase of the permeability in the plasma membrane of cells *in vitro* without affecting cell viability (14,15). They can also facilitate the transport of macromolecules through the stratum corneum into the viable epidermis and dermis (16,17).

To test the hypothesis that PW can disorganize the structure of a microbial biofilm and thus increase its permeability, fluorescence emission of the dye methylene blue (MB) was observed by confocal scanning laser microscopy following the application of the dye and a single PW on biofilms formed by *A. viscosus*, a species prominent in oral biofilms (18). In addition, preliminary studies investigated whether destruction of *A. viscosus* biofilms was possible after their sensitization with MB followed by exposure to a single PW and red light.

MATERIALS AND METHODS

Methylene Blue

MB (Sigma, St Louis, MO) was dissolved in Trypticase soy broth to give a solution at a concentration of 50 μg/ml and filter-sterilized immediately before use. The ultraviolet-visible absorption spectra of MB in 0.1 M NaOH/0.1% sodium dodecyl sulfate (SDS) as well as in Trypticase soy broth were recorded from 300 to 700 nm using quartz cuvets with 1 cm path length on a diode array spectrophotometer (model 8541A, Hewlett-Packard, Palo Alto, CA).

Biofilms

Bovine enamel surfaces measuring 5 × 5 × 2 mm were sterilized and then suspended in Trypticase soy broth with 5% sheep blood in wells of 12-well plates with cultures of *A. viscosus* T14 (S.S. Socransky, Forsyth Institute, Boston, MA). Plates containing the suspended enamel surfaces were incubated in an anaerobic chamber at 35°C and fresh medium with cultures of *A. viscosus* was added twice per week. Only the 0.7–0.8 mm thick biofilm which formed on the upper surface of the enamel after 8–10 days was treated and analyzed.

Generation of Photomechanical Waves

Biofilms were placed in a reservoir and 50 μg/ml MB was added (Fig. 1). A sterile black plastic (unexpanded polystyrene) target was placed over the top of the reservoir structure in contact with the solution (Fig. 1). The PW was generated by the ablation of the target material (19) with a Q-switched ruby laser (Laser Applications, Inc., Orlando, FL) and launched into

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ABBREVIATIONS: PW, photomechanical wave; MB, methylene blue; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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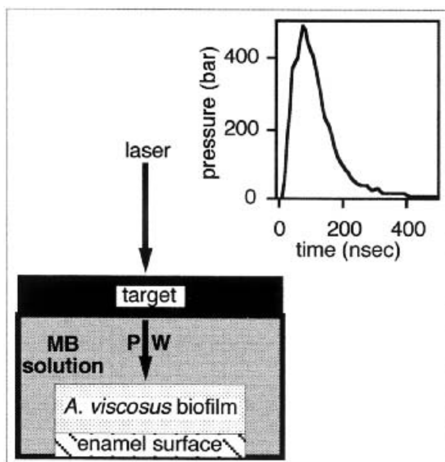

Fig. 1. The PW is generated by ablation of the target. The reservoir contains the MB solution which also acts as the acoustic coupling medium. Insert: the waveform of a PW generated by the ablation of black polystyrene with a single 23 ns Q-switched ruby laser pulse.

the reservoir containing the MB solution. The operating parameters of the ruby laser were 694.3 nm, 2.1 J, and 23 ns pulse duration. The laser pulse was delivered with an articulated arm and was completely absorbed by the target, so that only the PW (with a peak pressure of 600 bar, rise time of 50 ns and 110 ns in duration) propagated through the dye solution and impinged onto the biofilm. The beam size at the target was ~6 mm in diameter to achieve fluence of ~7 J/cm^2. The MB solution was allowed to remain in contact with the biofilm for 1 or 5 min after the application of the PW. Control biofilms were treated with MB in an identical manner, but they were not exposed to the PW. Subsequently, the MB solution was removed and the biofilms were washed once with sterile phosphate-buffered saline (PBS). Then they were placed in petri dishes covered with sterile PBS for confocal scanning laser microscopy studies.

Confocal Scanning Laser Microscopy

A Leica TCS NT confocal scanning fluorescence microscope (Leica Inc., Malvern, PA) equipped with a 10× water immersion objective lens was used to observe the fluorescence emission of MB. An argon laser (476 nm) was used as the excitation source for MB. Sections were collected at 100 μm intervals and these were then analyzed by image-processing techniques to assess the distribution of MB within the biofilm matrices.

Photodynamic Treatment

Biofilms were exposed to a single PW in the beginning of their incubation with 50 μg/ml MB for 1 or 5 min. Then, they were placed in petri dishes, washed once with sterile PBS and were exposed to light at 666 nm with fluence of 15 J/cm^2 at an irradiance of 50 mW/cm^2. An argon ion laser with an emission of 514.5 nm (Innova 100; Coherent Inc., Palo Alto, CA) was used to pump a dye laser (CR-599; Coherent) containing 4-dicyanomethylene-2-methyl-6-(p-dimethylamino-styryl)-4H-pyran dye (Exciton, Inc., Dayton, OH). The laser light was coupled into a 1 mm quartz fiber and appropriate spot sizes were created with an objective lens (no. 774317, Olympus, Tokyo, Japan). After illumination, adherent bacteria were scraped from the enamel surfaces and dispersed in Trypticase soy broth. Cell numbers were measured in a spectrophotometer (wavelength, 600 nm; 1 optical density unit equals approximately 10^9 cells/ml) in 1 ml tubes (20). Then serial dilutions were prepared and 100 μl aliquots were spread over the surfaces of blood agar plates. Survival fractions in each biofilm were calculated by counting the colonies on the plates and dividing by the number of colonies from dark controls incubated with the dye and kept at room temperature for periods equal to irradiation time. Other controls were: 1) Biofilms untreated with MB, light or PW, 2) Biofilms exposed only to a PW, 3) Biofilms exposed to light in the absence of MB, and 4) Biofilms exposed to light after incubation with MB.

RESULTS

Spectroscopy

The absorption spectra of MB in 0.1 M NaOH/0.1% SDS (wt/vol) were characterized by a Soret band maximum at 290 nm and a long-wavelength maximum at 666 nm (Fig. 2).

Penetration Depth of MB into Biofilms

Confocal scanning laser microscopy showed the penetration depth to be 400 μm for both incubation times 1 and 5 min. Application of a single PW increased the penetration depth to 630 μm and 700 μm respectively. Fig. 3 shows the penetration of MB (5 min incubation) in biofilms treated with a PW compared to controls.

Photodynamic Effects of MB

Incubation of the biofilms (approximately 10^9 bacteria per biofilm) in MB for 5 min followed by exposure to light killed 57% of the control bacteria and 99% of the bacteria exposed to the PW (Fig. 4). A one minute incubation in MB plus a PW and red light killed 96.5% of the bacteria (Fig. 4). Exposure to MB (1 min) and light produced only 34% killing (Fig. 4). There was no reduction in the viability of bacteria in biofilms after their exposure to MB or to a PW or to light alone compared to those of untreated biofilms (data not shown).

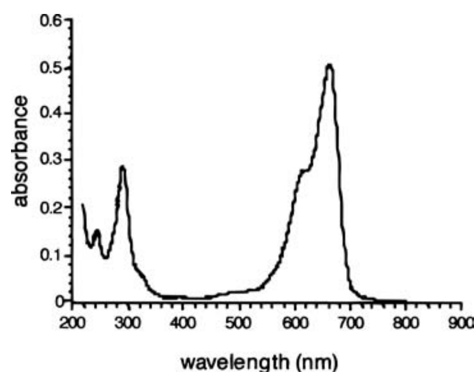

Fig. 2. Ultraviolet-visible absorption spectra of methylene blue in 0.1 M NaOH/0.1% SDS.

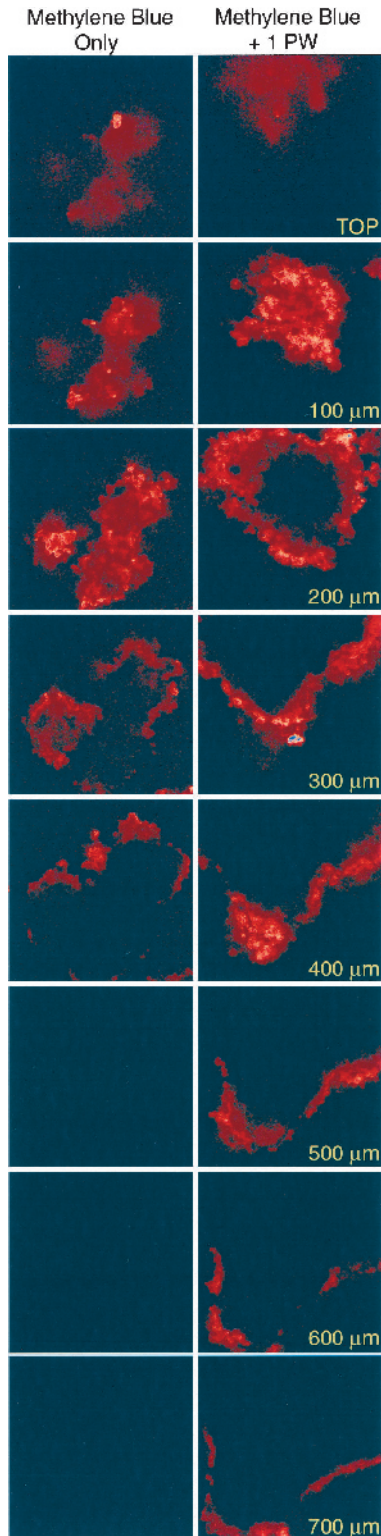

Fig. 3. Confocal fluorescence imaging of biofilms. There are two series of confocal images obtained by biofilms: exposed only to MB (left), and exposed to MB and a single PW (right). In the control, there is a fluorescent signal that extends from the top of the biofilm to a depth of 400 mm. No fluorescent signal was observed below 400 mm. In the specimen exposed to a PW the fluorescent signal extended to a depth of 700 mm.

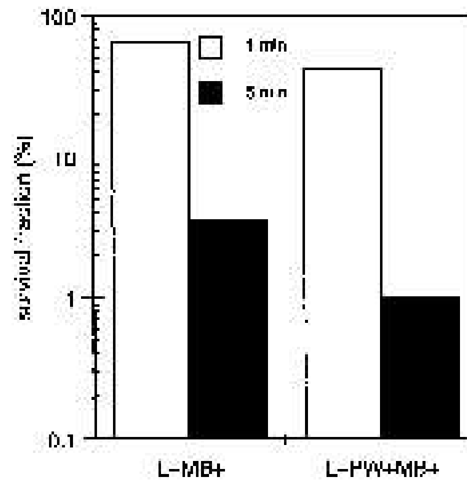

Fig. 4. Phototoxicity of *A. viscosus* in biofilms after incubation with 50 mg/ml MB for 1 min (white bar) and 5 min (black bar) followed by the application of a single PW and red light (L1PW1MB1) or light only (L1MB1). Each bar is the mean of values from two independent experiment (two biofilms per experiment).

DISCUSSION

This is the first report to show that the permeability of a microbial biofilm increases when it is exposed to a single PW. The results described here show that photomechanical waves could induce enhanced permeabilization of an oral microbial biofilm to a photoactivatable compound, an approach that enabled its photodestruction. We have selected MB for two reasons: a) It is an organic dye with fluorescent and photosensitizing properties (21), which has been shown to effectively inactivate many viruses and bacterial species after exposure to light (22,23), and b) The positive charge of the dye may promote its binding and effect on bacteria. Recently, it has been suggested that it was the charge borne by a cationic poly-L-lysine chlorin *e6* conjugate which was responsible for the increased uptake and pronounced phototoxicity of the conjugate on pure cultures of *Porphyromonas gingivalis* and *A. viscosus* (24).

Confocal microscopy has been used for the mapping of biofilm microstructures using fluorescent antibodies (25). Using the fluorescent probe MB (Fig. 3), confocal microscopy data suggest that the permeability barrier function of biofilms of *A. viscosus in vitro* could be modulated by PW. The increased biofilm permeability following a single PW indicates that diffusion of the positively charged MB could not be hindered by the matrix. It is possible that the matrix did not present any barrier to diffusion once all the binding sites had been filled with MB. The exact mechanism by which a PW enhances the penetration of MB through the biofilm is not known yet. Structural studies of mature biofilms by confocal microscopy demonstrated that they were composed of microcolonies of bacteria enveloped in a dense exopolysaccharide matrix interspersed with water channels (26), in which bulk fluid moves by connective flow throughout its very considerable extent (27). The PW may enhance fluid forces at the biofilm-bulk water interface that deform the microcolonies of bacteria and the matrix, so that fluid movement occurs. The great advantage of the present method is that it requires the presence of the drug for

1 to 5 min. The duration of the PW was only 110 ns. On the other hand, electric fields have been applied for 24 up to 48 h in order to enhance the efficacy of antibiotics in killing biofilm bacteria (9,10). In addition, ultrasonic irradiation has been shown to enhance the killing of *P. aeruginosa* in biofilms by gentamycin (8). The acoustic and any accompanying thermal effects of ultrasonic activation upon pure cultures of the periodontal pathogens *P. gingivalis* and *Actinobacillus actinomycetemcomitans* in an acoustically-simulated periodontal pocket model have been investigated (28). Statistically significant bactericidal effects in terms of viable counts were observed following 2.5 and 5 min sonication exposure times for *P. gingivalis* and *A. actinomycetemcomitans* respectively, but these effects were probably due to the incidental temperature changes of 47.6°C (118F) and 52.3°C (126F), respectively (28).

In this study the parameters of PW were: peak pressure at 600 bar, rise time of 50 ns and 110 ns in duration. The best combination of peak pressure, rise time and number of pulses has to be determined in the case of multi-species bacterial biofilms (e.g. dental plaque). The characteristics of PW (e.g., peak pressure, rise time) depend on the laser parameters (wavelength, pulse duration, fluence) and the optical and mechanical properties of the target material (e.g., polyimide, polystyrene, aluminum foil).

Our preliminary data indicate that the significantly increased concentration of MB after the application of a single PW on *A. viscosus* biofilms resulted in a pronounced light-induced inactivation of bacteria. It has been shown that biofilms of *S. sanguis* grown in a constant-depth film fermenter were less susceptible to photodestruction than planktonic cells after their incubation with toluidine blue and exposure to red light (29). No viable cells were detected after irradiation with a fluence of 96 J/cm^2 (28). It is known that biofilms in a constant-depth film fermenter form in a pre-set depth of 500 μm (30). In our study the thickness of *A. viscosus* biofilms was >700 μm and the total fluence delivered was only 15 J/cm^2. This clearly shows the contribution of PW to the enhancement of the penetration of biofilms by MB.

The approach of using PW described here could prove useful in delivering drugs into biofilms of oral species (caries, periodontitis, denture stomatitis, candidiasis) as well as into different biofilm types including those of *P. aeruginosa* (in the trachea of cystic fibrosis patients), *E. coli* (in urinary tract and intestinal infections), *Staphylococcus sp.* (on contact lenses). Further studies will explore the synergetic effect of PW and red light on biofilms consisting of single and multiple species. PW-assisted photodestruction of these biofilms will be compared with PW-assisted sensitivity to antiseptics and antibiotics.

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