MICROFLUIDIC BIOFILM OBSERVATION, ANALYSIS AND TREATMENT (MICRO-BOAT) PLATFORM

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ABSTRACT

We present the first demonstration of a novel bacterial biofilm treatment technique showing a 56% average decrease in bacterial cell viability compared to traditional antibiotic treatments in a Micro-BOAT platform. Integrated linear array charge-coupled devices achieve spatially realized optical density monitoring, correlating to both average biomass and localized biofilm morphology. For on-chip demonstration of biofilm treatment, a unique bioelectric effect using a superpositioned direct and alternating current electric field is applied in the presence of antibiotics. Use of the platform demonstrated successful real-time monitoring of biofilm treatment and validated an on-chip bioelectric effect showing a decrease in both bacterial cell viability and overall biomass.

INTRODUCTION

Bacterial biofilms are complex communities composed of a group of communicating bacteria and an extracellular matrix (ECM) [1]. The presence of the ECM limits molecular diffusion within the biofilm, therefore requiring higher doses of antibiotics for effective treatment [1]. In addition, since bacteria in close proximity can readily exchange genes such as those that promote antibiotic resistance, biofilms typically develop higher resistivity to antibiotics compared to planktonic bacteria [2]. Hence, once biofilms are established, their harmful toxin secretion can lead to severe infections that are difficult to treat, often requiring between 500-5000 times greater concentration of antibiotics compared to bacterial suspensions [3]. High doses of antibiotics are ill-advised, as they increase the risk of harmful side effects and the proliferation of multidrug resistant bacteria. Due to the high doses of antibiotics needed for biofilm treatment and the consequent emergence of multidrug resistant bacteria, a highly efficient biofilm treatment method utilizing low concentrations of antibacterial agents is of great interest.

When biofilms are treated by simultaneously combining antibiotics with electric fields, the synergistic effect results in dramatically improved biofilm treatment efficacy and is known as the bioelectric effect [3-5]. While this method has previously been shown utilizing electric fields exceeding biocompatible limits, recent work in our group has developed a new bioelectric effect capable of efficient biofilm treatment with parameters well suited for clinical applications [4, 7]. The new bioelectric effect utilizes a superpositioned DC and AC electric field based on the potential interactions of both field types in conjunction with low doses of gentamicin below the minimal inhibitory concentration (MIC) [8]. Demonstration of this method in a microsystem utilizing planar electrodes is critical to enabling future *in vivo* biofilm treatments in infected prosthetics and implants due to the necessary miniaturization of the system. The integration of this technique with microfluidics and optoelectronics enables the demonstration of the bioelectric effect at this scale.

In this work, we have developed a microfluidic biofilm observation, analysis and treatment (Micro-BOAT) platform for real-time spatiotemporal biofilm monitoring and treatment, allowing us to achieve the first demonstration of the enhanced

bioelectric effect in a micro-scale system. Detection of biofilm growth is achieved via the optical density (OD) properties of the biofilm as its density increases and has been demonstrated previously as a viable metric of biofilm development correlating to bacterial biomass [10]. The spatial resolution of the integrated charge-coupled devices (CCD) enables real-time tracking of localized biofilm morphology in the channel. End-point cell viability studies utilizing fluorescence microscopy further verify efficacy of the biofilm treatment. The use of the Micro-BOAT platform enables characterization of the bioelectric effect in environments representative of *in vivo* applications using a non-invasive monitoring technique, while simultaneously providing a low cost, high throughput means of treatment evaluation.

METHODS

Micro-BOAT Platform

Optical density monitoring of biofilm growth in a microfluidic channel has been previously validated in our group [10]. This device utilized individual commercial-off-the-shelf (COTS) photodiodes to detect biofilm OD at two locations within the microfluidic chamber. Based on this work, the Micro-BOAT platform is an integrated microsystem consisting of both COTS components as well as microfabricated elements to achieve full-channel analysis of biofilm optical density. CCD devices and supporting electronics are featured in parallel on a 9.5cm x 8.1cm printed circuit board (PCB) to enable six parallel experiments on a single chip. Microfluidic chambers for biofilm growth and electrode structures for applying the enhanced bioelectric effect are constructed using microfabrication techniques on a separate module of the platform. A schematic of the microsystem is provided in figure 1. The CCDs (TAOS TSL202R) feature 128 x 1 linear pixel arrays with individual photodiodes measuring 120 μ m (H) by 70 μ m (W) spaced 55 μ m apart, thereby spanning an overall array length of 1.6cm. The microfluidic module is comprised of molded polydimethylsiloxane (PDMS) channels bonded to a transparent PyrexTM substrate to allow for light transmission to the CCD photopixels.

Figure 1: Cross-sectional schematic of the integrated Micro-BOAT platform with CCD components. Electric contacts introduce the enhanced bioelectric effect to biofilms within the microfluidic channel.

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Gold electrodes patterned on the PyrexTM substrate provide an electric field for the bioelectric effect while simultaneously limiting peripheral light from disrupting optical density measurements. The electrodes are spaced 2mm apart along the full length of the microfluidic channel. The generated electric field is therefore perpendicularly in-plane to the direction of fluid flow in the channel, with the resultant electrophoretic force being orthogonally out-of-plane with respect to the flow direction. The linear photopixel arrays of the CCD/PCB module are aligned to the channels of the microfluidic/electrode module to enable optical density measurement. Uniform illumination of the Micro-BOAT sensors, necessary for proper device calibration, is achieved through a diffused LED light panel tuned to 630 nm by a polycarbonate lighting gel film in order to match the peak sensitivity of the CCD devices.

The microfluidic device module of the Micro-BOAT platform is fabricated via the process shown in figure 2.

Figure 2: Fabrication process flow for the microfluidic and electrode module of the Micro-BOAT platform. The direction of fluid flow within the micro-channels is shown perpendicular to the page.

A mold of the microfluidic structures is fabricated by patterning 100µm-thick KMPR-1050 on a silicon substrate using contact photolithography. To avoid adhesion of PDMS to the channel mold during curing, vapor phase silanization in a vacuum dessicator is performed for one hour using trimethylchlorosilane (Silane M3). Using this technique, a single mold can be reused multiple times without quality reduction in the PDMS channels. PDMS (Sylgard 184, Dow Corning) in a 10:1 ratio is then poured over the mold and cured in a furnace for 20 minutes at 80°C. After cooling, the PDMS is removed from the mold and cut to fit the PyrexTM substrate. To fabricate the gold electrodes for the bioelectric effect, AZ-5214 photoresist is initially patterned on a PyrexTM substrate using contact photolithography to achieve a 1.6µm thickness profile. E-beam evaporation of Cr/Au (15nm / 200nm) and liftoff is then performed to complete the electrode structures. Finally, the PDMS is reversibly bonded to the gold-patterned substrate by applying methanol to the PDMS, then aligning and placing it onto the PyrexTM chip. Here, reversible bonding is preferred to allow for disassembly, cleaning, and reuse of the patterned substrate for multiple experiments. The resulting microfluidic chambers measure 100µm deep, 2000µm wide, and

1.75cm long. The microfluidic chamber is integrated with external Tygon tubing via interface ports that are drilled in the PDMS using a 2mm diameter dermatological punch.

Bioelectric Effect

A combinatorial biofilm treatment using electric fields and antibiotics, known as the bioelectric effect, has been demonstrated to improve efficacy of antimicrobial drugs [3-6]. Since the treatment was first demonstrated in 1994 [3], studies have utilized either a direct or alternating current (DC or AC) electric field to achieve the bioelectric effect [3-6]. As electric fields are applied along with antibiotics, the fields can induce a pH gradient and increase antibiotic permeability into the biofilm [4]. The bioelectric effect has also demonstrated biocidal characteristics with different antibiotics [6]. However, a successful clinical application has not yet been realized due to remaining challenges, including electrolysis of the media as a result of the high electric fields applied for the effect. When the intensity of the electric potential is greater than the limit voltage (0.8V), electrolysis of the surrounding media results in the generation of toxic molecules such as hydrogen ions, thus interrupting cell metabolism and resulting in the death of surrounding healthy cells [9].

Recent work in our group has developed an enhanced bioelectric effect capable of efficient biofilm treatment with parameters well-suited for clinical applications [7]. The intensity of the electric field is characterized by using an electric field potential below the electrolysis threshold of the media (0.8V), which corresponds to 2 V/cm for this platform. By avoiding electrolysis induction, this treatment method can be directly applied to clinical applications. In the experiments, we introduced a superpositioned DC and AC field with gentamicin based on the potential synergistic effects of different electric fields [7]. The DC component of the superpositioned field was characterized to avoid electrolysis induction [7]. The frequency of the AC component was selected based on literature [4, 5] and the magnitude of the field matched to the DC component. Thus, the superpositioned electric field was composed of a 1.25 V/cm sinusoidal signal at 10 MHz (AC component) with a 1.25 V/cm DC offset (DC component). The concentration of gentamicin (10µg/mL) was selected to be lower than the minimal inhibitory concentration (MIC) ($\approx$ 32µg/mL) [8] to minimize the concentration of antibiotics. In doing so, the bioelectric effect has a critical advantage, by deterring the development of antibiotic resistive bacterial strains.

Experimental Procedures

The demonstration of the superpositioned bioelectric effect in a micro-scale device has been achieved using the Micro-BOAT platform. Bacterial cultures of *Escherichia coli* BL21 pGFP are grown in Lysogeny Broth (LB) media to an OD₆₀₀ of 0.25 AU. Testing is performed by initially inoculating a microfluidic chamber with bacterial suspension for 2 hours at 37°C with no flow to allow microbial attachment to occur on the channel substrate. LB media is then flowed continuously through the channel for 24 hours at 20µL/h by a syringe pump (Cole-Parmer Instrument Company, USA) to replenish nutrients and foster biofilm growth. For the channel dimensions presented here, this equates to an effective flow velocity of 30µm/s and has been optimized to provide an environment conducive to biofilm growth. Following the 24 hour growth period, four treatments are performed on the mature biofilms: (1) control (no antibiotic or electric field), (2) only superpositioned electric field, (3) only gentamicin, and (4) enhanced bioelectric effect (superpositioned electric field with gentamicin). Control biofilms are continuously provided LB media at the same flow rate in order to determine steady-state biofilm growth. Biofilms treated with gentamicin, both

in antibiotic-only experiments and those featuring the enhanced bioelectric effect, are done so with a 10 μ g/mL concentration of the drug [7]. The electric field featured in the biofilm experiments was introduced by a function generator (Agilent Inc., USA) [7]. Each of the four biofilm treatments is conducted with 3 experiment samples to demonstrate repeatability of the various assays.

Optical density measurements are taken non-invasively in real-time with respect to both average OD change as well as localized morphology. Measurements are obtained every 8 minutes from the CCD devices to monitor the spatiotemporal development of the bacterial biofilms beginning after the initial two-hour inoculation period. Additionally, bacterial cell viability staining and fluorescence microscopy is used to provide end-point analyses of cell viability within the biofilms to verify treatment efficacy. Since the strain of bacteria used (BL21 pGFP) fluoresces green when the bacteria are metabolically active, only propidium iodide (PI) (Invitrogen Inc., USA) is needed to stain dead bacteria allowing them to fluoresce red. Following biofilm treatments, the microfluidic chamber is initially rinsed with phosphate buffered saline (PBS) solution at a rate of 200 μ L/h for one hour to quantify only adherent bacteria. The stain is then supplied at a concentration of 1.5 μ L PI per 1000 μ L PBS at a flow rate of 200 μ L/h for an additional 2 hours and unabsorbed stain rinsed from the microchannel using PBS at the same flow rate for an additional one hour. The ratios of live bacteria surface coverage with respect to the total biomass surface coverage for different treatments are analyzed using the software package ImageJ (Image J 1.44, USA). For each experiment, both optical density and viability analyses are performed to confirm the OD method for biofilm monitoring

RESULTS AND DISCUSSION

Optical Density Monitoring

Figure 3 presents the average change in biofilm OD along the entire microfluidic channel for each of the four treatments, normalized to their initial optical densities. Before applying treatment, mature *E. coli* biofilms are formed in the microfluidic channels for 24 hours, as described previously. Each treatment is then performed continuously for an additional 24 hours. As shown in figure 3, the control and electric field treatments resulted in overall increases in biofilm optical density, corresponding to bacterial biomass growth. In contrast, those biofilms treated with gentamicin and the enhanced bioelectric effect showed decreases in biomass over the course of the treatments. After treatment, biofilms subject to the bioelectric effect experienced a net 40% decrease in OD which implied substantial biomass decrease, while the control case demonstrated a 260% increase in OD as shown in figure 4(a). The opposing trends in OD change between the control and bioelectric effect demonstrate that the combination of a superpositioned electric field in the presence of antibiotics significantly improves biofilm treatment. Additionally, figure 4(b) illustrates the capabilities of the microsystem to monitor localized biofilm morphology in real-time without conventional instruments to get this image such as confocal microscope, a critical advantage of this platform. Spatial understanding of the biological system aids in filtering of signal disturbances due to free-flowing particulates in the microchannel, thus enabling more accurate biofilm analyses.

Biofilm Bacteria Viability

In addition to the biomass change monitoring by OD measurements, efficacy of each of the biofilm treatments was evaluated using a live/dead cell staining method to verify the treatment efficacies. Dead cells are stained red with propidium iodide, and viable cells appear green due to the expression of GFP.

Figure 3: Measured changes in OD during biofilm treatment using the Micro-BOAT platform. Each curve represents the average OD change for three samples with standard deviations shown at representative time points.

Figure 4: (a) Relative average changes of biomass after treatments in the microfluidic channel. Biofilms treated with the bioelectric effect had a decrease of approximately 40% in overall biomass while control and electric field treatments resulted in biomass increases of 260% and 140% respectively. (b) Surface reconstruction of biofilm morphology changes in the channel over time. Waterfall images are reconstructed using MATLAB based on the OD from the CCDs. The result illustrates the capability to investigate localized biofilm morphology differences.

Figure 5 shows bright field total fluorescence (left column) and only green fluorescence (right column) for each of the four treatments. The total fluorescence images represented the overall biomass in the microfluidic channel. As shown in figure 5(g), biofilms treated with the superpositioned bioelectric effect showed the least amount of bacterial biomass, demonstrating the enhanced effect of this biofilm treatment compared to the other methods. Viable bacteria within the biofilms were inspected using a green fluorescence filter with results shown in the right column of figure 6. Biofilms treated with the bioelectric effect show a lower density of live cells compared to other treatments, thus demonstrating the capability of this method to reduce both overall bacterial biomass as well as cell viability. A quantitative analysis of viable biofilm bacteria was performed using the image processing program ImageJ. The percentage of viable bacteria in each treatment was calculated based on the surface coverage of the green fluorescence images with respect to the coverage of total fluorescent biomass (figure 5). Thus, this approach represents the percentage of viable bacteria within a given biomass, which is clinically important.

Figure 5: Representative fluorescence microscopy images of biofilms in the channel after treatment. Biofilms treated with the bioelectric effect showed less biomass (left column) as well as low live bacterial density (right column).

Figure 6: Results of cell viability studies. Using the bioelectric effect treatment, the density of dead cells was increased an average of 56% compared to the antibiotic treatment.

As shown in figure 6, the bioelectric effect shows 56% improved treatment efficacy compared to only antibiotic treated biofilms.

Based on the results in figures 3-6, the on-chip bioelectric effect demonstrates not only decreased biomass growth, but also an increased biocidal effect to biofilm bacteria. The effective use of the enhanced biofilm treatment using planar electrodes facilitates the development of a micro-scale biofilm treatment system, allowing this concept to be applied to clinical applications.

CONCLUSIONS

The successful treatment of biofilms using the bioelectric effect for improved antibiotic efficacy is demonstrated in the Micro-BOAT platform. Using the platform, parallel biofilm treatment experiments were conducted to determine the relative efficacy of each treatment. The demonstration of the bioelectric effect at the micro-scale lays the foundation for future in-vivo bacterial treatment concepts. Furthermore, the unique capability of the CCD-enhanced Micro-BOAT platform to perform temporally and spatially resolved monitoring makes it useful for future drug discovery studies.

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