RESEARCH LETTER

A surface with a biomimetic micropattern reduces colonization of *Mycobacterium abscessus*

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Abstract

Nontuberculous mycobacteria (NTM) are ubiquitous organisms found in soil, water, and biofilms. Engineered surface topography has been proposed as a method to reduce microbial biofilm formation. The Sharklet® micropattern silicone surface has been shown to reduce biofilm formation of pyogenic bacteria. We hypothesized that this micropattern surface will also reduce colonization by *Mycobacterium abscessus*, a human pathogen. Smooth and micropattern silicone samples were incubated with 1×10^6 *M. abscessus* mL^{-1} for 2 and 4 days. After processing to optimize recovery of adhered mycobacteria, there was a 75% and 50% reduction in the number of viable *M. abscessus* recovered from the micropattern surfaces compared to the smooth surfaces at 2 and 4 days after inoculation, respectively. Ziehl–Neelsen staining after measures to remove the adherent microorganisms revealed fewer residual *M. abscessus* on the micropattern samples as compared to smooth samples, validating the quantitative culture results. Microscopic observation of 2, 4, and 8 day *M. abscessus* cultures on micropattern samples showed that the organisms preferentially colonized within the channels between the rectangular features. In summary, a micropattern surface reduces the colonization of a pathogenic NTM. It remains to be seen whether this micropattern can reduce infections in humans.

Introduction

Nontuberculous mycobacteria (NTM) are opportunistic, pathogenic bacteria found in soil, natural and potable water, and in biofilms that form on water-solid interfaces (Falkinham, 2009). NTM cause skin and soft tissue infections, chronic lung disease, and extrapulmonary, and disseminated infections (Chan & Iseman, 2013). The incidence and prevalence of NTM lung disease are increasing in the United States and many parts of the world (Marras & Daley, 2002; Cassidy et al., 2009; Griffith & Stout, 2010; Prevots et al., 2010; Winthrop et al., 2010). Genetic studies have linked NTM isolated from patients with the same organisms isolated from potable water sources (von Reyn et al., 1994; Aronson et al., 1999).

Nontuberculous mycobacteria can form biofilms on man-made surfaces that come into contact with water, a process that can occur in as few as 2–3 days (Uttley & Simpson, 1994; Hall-Stoodley et al., 2006; Howard et al., 2006). Biofilms containing high density of mycobacteria are potential sources of NTM infections in humans. In showerhead biofilms, NTM concentrations have been found to be 100-fold greater than in water (Feazel et al., 2009). NTM associated with biofilms are also more resistant to disinfectants than planktonic NTM (Donlan & Costerton, 2002; Carter et al., 2003). Compared to planktonic cells, *Mycobacterium avium* isolated from biofilms are more resistant to killing by the disinfectants chlorine, potassium monopersulfate, and chloroheximide acetate (Carter et al., 2003). Despite having undergone sterilization protocols, fiberoptic bronchoscopes, and video-assisted surgical

instruments contaminated with NTM biofilms have caused clinically significant infections in patients (Pappas *et al.*, 1983; Duarte *et al.*, 2009). Thus, reducing NTM colonization and subsequent biofilm formation may not only decrease potential sources of infections but may also reduce resistance of NTM to disinfectants (Bardouniotis *et al.*, 2001).

Due in part to slow growth properties and waxy, lipid-rich outer covering, NTM are innately resistant to many antibiotics and treatment typically requires a cocktail of antibiotics for prolonged periods (Griffith *et al.*, 2007; Kasperbauer & Daley, 2008). Despite intensive treatment, it is estimated that only 55% of patients with NTM lung disease are successfully treated and that the disease recurs in nearly one-third of those with presumed cure (Field *et al.*, 2004; Kobashi & Matsushima, 2007; Hoefsloot *et al.*, 2009; Jarand *et al.*, 2011). One factor that can help determine if the initial NTM infection becomes established is whether biofilms form on tissue surfaces (Yamazaki *et al.*, 2006; Rose & Bermudez, 2014). NTM associated with biofilms are also more resistant to antibiotics than planktonic organisms (Donlan & Costerton, 2002; Greendyke & Byrd, 2008). For example, amikacin and clarithromycin only had modest effects at high concentrations (up to 512 μg mL^{-1}) on *M. abscessus* biofilms whereas the planktonic form was susceptible (< 8 μg mL^{-1}) to both drugs (Greendyke & Byrd, 2008).

Strategies to reduce biofilm formation could significantly reduce exposure to high concentrations of potentially pathogenic bacteria. A number of surfaces have been engineered to reduce bacterial biofilm formation, including liquid-infused structured surfaces (Epstein *et al.*, 2012) and surfaces with altered topography (Schumacher *et al.*, 2007). The Sharklet® micropattern surface mimics the texture found on shark skin and has been shown to reduce colonization and biofilm formation of bacterial pathogens (Chung *et al.*, 2007; Reddy *et al.*, 2011). Chung *et al.* (2007) found that this micropattern surface significantly reduced biofilm formation of *Staphylococcus aureus* as compared to a smooth control surface. Similarly, the micropattern surface reduced *Escherichia coli* colonization by 47% and colony size by 77% (Reddy *et al.*, 2011). Because NTM frequently form biofilms and acquisition of NTM infection is largely due to exposure to water sources, we investigated whether the Sharklet® micropattern surface reduces colonization of *M. abscessus*.

Materials and methods

Materials

Twelve-millimeter-diameter and 0.4-mm-thick silicone samples (Silastic T2, Dow-Corning) were manufactured by Sharklet Technologies, Inc. (Aurora, CO) with a smooth surface or a Sharklet® micropattern (Chung *et al.*, 2007). The topography of the micropattern surface is comprised of 2-μm wide rectangular features of varying lengths (4–16 μm) and 3 μm in height using deep reactive ion etching (Fig. 1). The features are combined into a repeating diamond-shaped pattern with fixed spacing between neighboring features (Chung *et al.*, 2007). *Mycobacterium abscessus* ATCC strain No. 19977 was grown in Middlebrook 7H9 broth containing OADC enrichment (Sigma-Aldrich, St. Louis, MO). Lipase from *Pseudomonas cepacia* was purchased from Sigma-Aldrich.

Biofilm formation

For each experiment, eight silicone samples (four smooth and four micropattern) were dipped in 95% ethanol and suctioned to the bottom of a 100 × 15 mm polystyrene petri dish and sterilized with ethylene oxide. Each petri dish was filled with 20 mL of Middlebrook 7H9 broth containing OADC enrichment and 10^6 *M. abscessus* mL^{-1} and incubated at 37 °C for 2, 4, or 8 days.

Biofilm removal

After 2 and 4 days of incubation, the supernatant was discarded and the silicone samples were rinsed gently three times with sterile 1X phosphate-buffered saline. To reduce the variance introduced by nonpattern edges, the edges of each silicone sample were removed using a sterile 8-mm skin biopsy punch (Miltex, York, PA) (Reddy *et al.*, 2011). To disrupt the integrity of the biofilm and facilitate its subsequent removal, the punch-outs were transferred to 15-mL conical tubes containing lipase
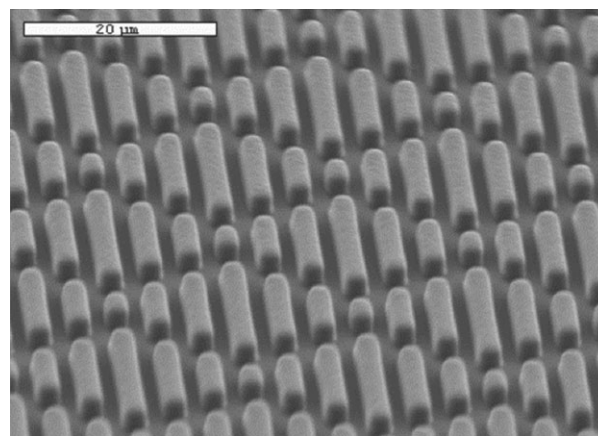
Fig. 1. Topography of the micropattern samples. Scanning electron microscopy of the Sharklet® silicone micropattern showing the topography of raised features in a repeating diamond-shape pattern.

buffer (0.1 M Tris-HCl, pH 5.5, 0.11 M NaCl, 0.11 M CaCl$_2$, 1 mg mL^{-1} lipase) (Thorel *et al.*, 1991), vortexed for 30 s, incubated for 1 h at room temperature, vortexed for another 30 s, and then 1 mL 0.25% SDS + 1 mL Middlebrook 7H9 plating broth with OADC enrichment added. The tubes were again vortexed for 30 s, and then sonicated at 50/60 Hz in a Bransonic 220 ultrasonic bath for 5 min to further enhance biofilm disruption and bacteria removal. After sonication, the tubes were vortexed for 30 s, 100-μL aliquots were removed for serial dilution with 7H9 broth, and the diluted samples were plated in duplicate onto 7H10 Middlebrook agar plates. Sonication did not reduce the colony-forming ability of *M. abscessus*. The plates were incubated for 3–5 days at 37 °C until distinct colonies were observed, colony-forming units (CFU) counted, and CFU mL^{-1} calculated.

Staining

To evaluate cells left on surfaces following *M. abscessus* removal, each silicone sample was heat-fixed to a glass slide and underwent Ziehl–Neelsen staining to qualitatively assess the number of mycobacteria still remaining on the surfaces.

Statistics

Mean CFU was analyzed using the unpaired Student's *t*-test, and statistical significance was defined as $P < 0.05$. Percent reductions were determined using the equation: $100 \times (\text{mean CFU}_{\text{smooth}} - \text{mean CFU}_{\text{micropattern}})/\text{mean CFU}_{\text{smooth}}$.

Results

Two days after inoculation of the silicone samples, there was a statistically significant, 75% reduction in the number of *M. abscessus* CFU recovered from the micropattern samples as compared to the smooth samples, with mean *M. abscessus* concentrations of 8.3×10^2 CFU mL^{-1} and 3.3×10^3 CFU mL^{-1}, respectively (Fig. 2a). Four days after inoculation, there was a statistically significant 52% reduction in the number of viable *M. abscessus* on the micropattern surface as compared to the smooth samples, with mean *M. abscessus* concentrations of 1.4×10^3 CFU mL^{-1} and 2.9×10^3 CFU mL^{-1}, respectively (Fig. 2b).

If the biofilm recovery process did not adequately or uniformly remove *M. abscessus* from the micropattern samples, this could account for fewer mycobacteria recovered. To evaluate this possibility, both types of silicone surfaces were stained using the Ziehl–Neelsen procedure after lipase and sonication treatment. The number of residual mycobacteria was visibly reduced on the
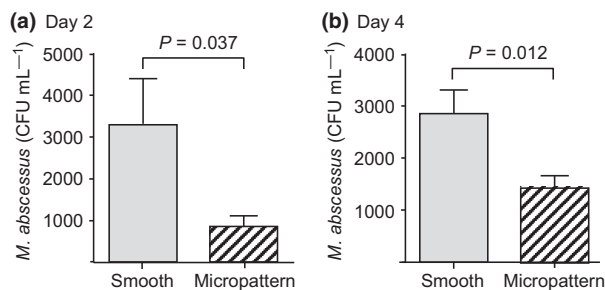

Fig. 2. Quantitation of *Mycobacterium abscessus* recovered from smooth and micropattern silicone samples. (a) Smooth and (b) micropattern silicone samples were inoculated with *M. abscessus* for 2 and 4 days; adherent mycobacteria were removed from the silicone samples and then cultured. Data shown are the mean ± SEM of three independent experiments for 2 day culture and five independent experiments for 4 day culture.

micropattern surfaces as compared to the smooth surfaces (Fig. 3), further supporting the CFU enumeration results.

To determine if *M. abscessus* preferentially populate specific features of the micropattern topography – which can provide an indication of the potential mechanism by which there is less colonization – we observed by microscopy day 2, 4, and 8 *M. abscessus* culture (10^6 *M. abscessus* mL^{-1}) on the micropattern and smooth surfaces without performing the biofilm removal process. As shown in Fig. 4, *M. abscessus* preferred to colonize within the channels between the rectangular features of the micropattern surface rather than on top. At all three time points examined, there were noticeably fewer *M. abscessus* on the micropattern surface than on the smooth surface.

Discussion

Biofilms are an important threat to human health as they may harbor large numbers of pathogenic bacteria. Up to 80% of bacterial infections in humans involve microorganisms from biofilms, and biofilm formation on medical devices can lead to nosocomial infections and potentially higher mortality rates (Renaud & Brun-Buisson, 2001; Duarte *et al.*, 2009). Thus, reducing biofilm formation in potable water systems could be an effective method to reduce exposure to harmful bacteria. Innovative biofilm-resistant water treatment facilities, water mains, and household plumbing could reduce exposure to waterborne, opportunistic pathogens such as mycobacteria and legionella. Given the current situation with antibiotic-resistant bacteria, preventing exposure is an attractive approach to combating infections. In this study, we found that a micropattern surface reduces the colonization of *M. abscessus*, a pathogenic environmental bacteria known to cause lung disease and soft tissue infections.
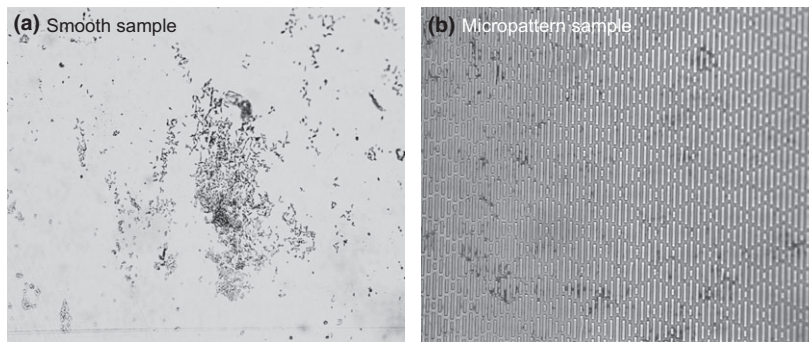
Fig. 3. Ziehl–Neelsen staining of samples after bacterial recovery process. After the smooth and micropattern samples were subjected to measures to remove adherant *Mycobacterium abscessus*, the samples were stained by the Ziehl–Neelsen method. Shown are representative smooth and micropattern samples of three independent experiments. Original magnification = 400×.
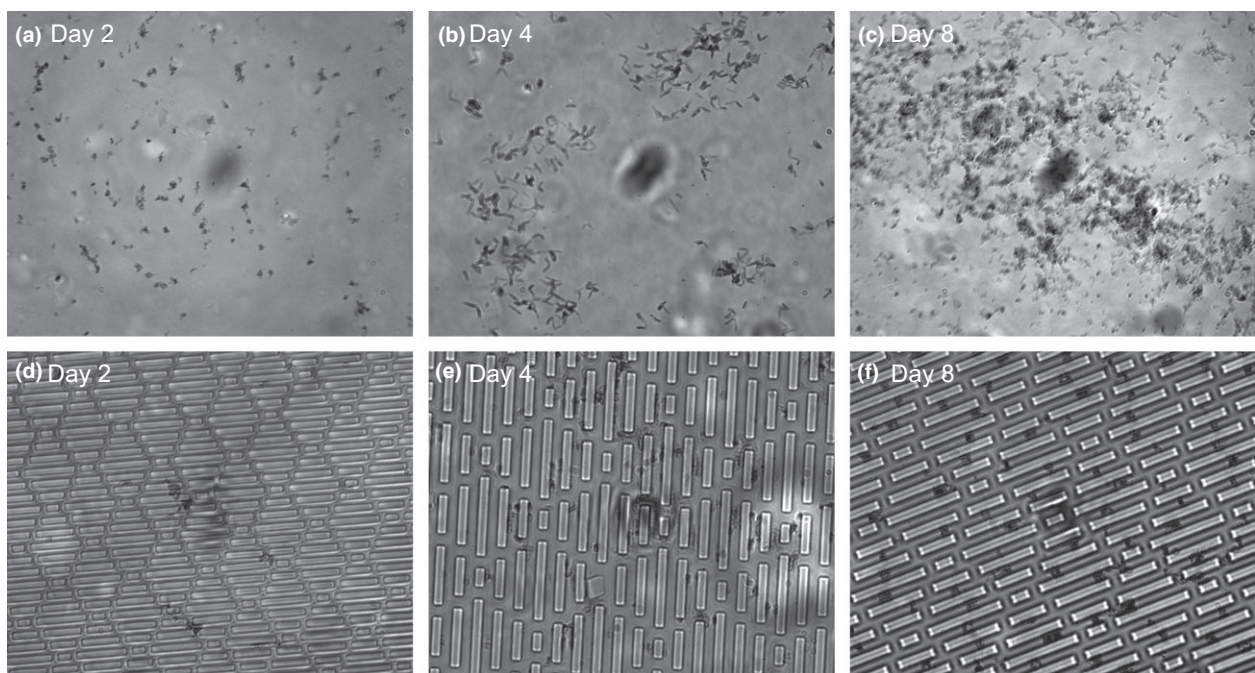
Fig. 4. Ziehl–Neelsen staining of *Mycobacterium abscessus* culture on smooth and micropattern samples with no bacterial recovery process. *Mycobacterium abscessus* were cultured on (a–c) smooth and (d–f) micropattern surfaces for 2, 4, and 8 days. At the indicated durations of incubation, the supernatants were discarded, the silicone samples gently rinsed thrice with 1X phosphate buffer saline, heat-fixed on glass slides, and stained by the Ziehl–Neelsen method. Representative microscopic fields of two independent experiments are shown. Original magnification of a, d = 400× and of b, c, e, f = 1000×.

What are some potential mechanisms for the reduced colonization? One possibility is that the raised surface of the micropattern results in reduced surface area for the mycobacteria to adhere to if the channels between the features failed to fill with growth medium. However, a previous study with *S. aureus* showing the presence of bacteria within the channels would argue that bacteria-containing liquid medium is able to fill the channels. Another possibility is that the micropattern surface may

inhibit migration when bacteria encounter topographic features which are perpendicular to the overall direction of migration, as shown previously for *E. coli* (Reddy *et al.*, 2011). Indeed, microscopy showed that on the micropattern surface, *M. abscessus* were preferentially localized within the channels between the rectangular features. In addition, microscopy showed that *M. abscessus* organisms on the smooth surface were more clumped whereas those on the micropattern surface appeared less

aggregated, *that is*, more individual cells. This may account for why there was an increase in CFU cultured from the micropattern surface between 2 and 4 days whereas the number of CFU remained relatively stable for the smooth surface over this period. Microscopic studies also showed no evidence of 'overflow' of *M. abscessus* above the channels over the 8-day period. Another potential mechanism is reduced bioadhesion of *M. abscessus* to the micropattern surface. Bioadhesion is a complex and species-specific process which is influenced by surface factors including surface tension and elasticity as well as bacterial processes such as adhesive proteins, lipids, and polysaccharides (Carman *et al.*, 2006; Decker *et al.*, 2013). By manipulating the surface microtopography, surface tension and the angle at which the bacteria contact and adhere to the surface can be altered. For example, if a bacterial cell is too large or too long, it must bridge or otherwise conform to the shape of the micropattern. *Mycobacterium abscessus* are relatively long microorganisms (typically 0.5-μm wide and 1.0–2.5-μm long), and thus, if some organisms lie across more than one rectangular feature, such bridging can reduce the area of contact between surface and bacteria, effectively reducing both overall adhesion strength and the potential for bacterial settlement. However, this 'bridging' mechanism appears unlikely as there was essentially no bacteria that colonized the top surface of the features or 'straddled' more than one feature, unlike what has been observed for settlement of a range of marine organisms (Decker *et al.*, 2013). Prior work has demonstrated that micropattern surfaces demonstrated altered hydrophilic properties by mechanically changing the surface energy (Carman *et al.*, 2006). In that study, micropattern surfaces lowered settlement density of motile algae spores by about 86% relative to smooth surfaces. Thus, although the precise mechanism(s) for reduced *M. abscessus* colonization is not known, the current evidence indicates that reduced surface area, active physical constraints within the channels, and/or reduced bioadhesion are potential mechanisms by which the micropattern surface results in fewer adherent mycobacteria.

Why *M. abscessus* does not colonize on the top surfaces of the features remains to be determined. A plausible explanation is that Brownian motion prevented the organisms from adhering effectively to the relatively narrow top surface of the rectangular features resulting in gravity pulling the organisms to the bottom of the channels.

Further investigations are needed to determine the specific characteristic(s) of the micropattern that is responsible for reduced mycobacterial colonization. While surfaces lined by this micropattern may be more resistant to colonization and biofilm formation, it remains to be seen whether the use of such engineered surfaces on medical devices or plumbing system translates to reduced community and hospital-acquired infections.

Acknowledgements

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