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Effects of disordered hemispherical micropatterns on *Staphylococcus epidermidis* biofilm formation

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ABSTRACT

Surfaces which have physical patterns in the scale of bacteria cells have been shown to influence the microorganism's adhesion and biofilm formation characteristics. Layer-by-layer self-assembly was utilized to create disordered hemispherical patterns on poly(dimethylsiloxane) with a feature size of 0.5 μm, 1.0 μm and 2.0 μm. The effects of pattern size on the retention and biofilm formation of *Staphylococcus epidermidis* were examined as a function of culture time. The 1.0 μm pattern significantly reduced biofilm surface coverage by ~30% after 5 h of culture in comparison to that on an unpatterned surface while the effect of the 0.5 and 2.0 μm patterns was negligible. On the 1.0 μm surface, bacteria initially adhered on the unpatterned areas of the disordered surface and subsequently developed into biofilms by spreading across the unpatterned areas while avoiding those covered by the pattern. The results suggest that the size of surface patterns is an important factor in altering bacteria adhesion and biofilm formation characteristics.

1. Introduction

When an implant is surgically introduced into the human body, bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis* arrive at the implant surface before host cells [1]. With the lack of immunodefense mechanisms in place at the implant surface, opportunistic bacteria are able to adhere to the surface and subsequently form biofilms. It is generally believed that the extracellular matrix of the biofilms protect bacteria from both host defenses and antibiotics, thereby making implant-associated infection difficult to treat [2–4]. Therefore, there has been significant interest in producing an implant surface which is resistant to bacteria adhesion and biofilm formation as an avenue of reducing the susceptibility of implant-associated infection.

Much work has been done studying how surface topography or physically patterned surfaces interact with cells to alter their adhesion and proliferation characteristics [5,6]. It appears that the bacterial response can be influenced by the shape, order, symmetry, and total area covered by a pattern as well. One possible mechanism for preventing bacteria attachment via surface patterning takes advantage of physical contact [7,8]. The formation of focal contacts can be prohibited by using surface patterning in the size range of the microorganism of interest. Bacteria are then forced to span the distance between structures in the manner of a bridge. This results in reduced contact area, equating to overall lower adhesion strength.

With recognition of the spherical shape and the ~0.7 to 1 μm diameter of *S. epidermidis*, the aim of this investigation was to study the potential effects of hemispherical pattern features in the size range of 0.5–2.0 μm on *S. epidermidis* biofilm formation. Layer-by-layer self-assembly (LBLSA) was used to create disordered, hemispherical pattern features by: (1) self-assembling a monolayer of silica particles of the desired size on a silicon substrate and (2) using the silica particle self-assembly as a mold to pattern polymer surfaces. Poly(dimethylsiloxane) (PDMS) was used as a model polymeric material since it is biocompatible and inexpensive. LBLSA was chosen because of its versatility as a non-line-of-sight technique [9], allowing the possibility of patterning three-dimensional implant surfaces that planar techniques (e.g., optical and electron beam lithography) are unable to pattern. However, the technique is limited to creating disordered patterns.

2. Experimental

2.1. Preparation of silicon substrates

Silicon substrates were produced from a (1 0 0) wafer with one polished side. A diamond tipped etching device was used to break the wafer into several pieces approximately 1 cm × 3 cm. The pieces were then rinsed with deionized water (resistance 18.2 MΩ, Millipore, Bedford, MA, USA) to remove any debris left on the substrate. Using a glass container under a chemical hood, the substrates

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were cleaned using a piranha solution. Substrates were placed in the container flat with the polished surface upward. H_2SO_4 (95–98%, Sigma–Aldrich, St. Louis, MO, USA) and H_2O_2 (35 wt.%, Sigma–Aldrich) were added to the container at a ratio of 3:2 to cover the substrates. The container was allowed to sit for 1 h. Using tweezers the samples were removed and rinsed thoroughly with dionized water and placed in a clean Petri dish filled with dionized water. The cleaning solution was then neutralized with sodium hydroxide (NaOH) and diluted with water.

2.2. Application of polyelectrolyte multilayer

A dipping robot (Dipping Robot DR-3, Riegler & Kirstein GmbH, Berlin, Germany) was used to apply the polyelectrolyte multilayer (PEM). The polyelectrolytes used were poly(diallyldimethylammonium chloride) (PDDA, molecular weight ~100,000–200,000, 20 wt.% solution, Sigma–Aldrich) and poly(styrenesulfonic acid sodium) (PSS, molecular weight ~70,000, salt, Alfa Aesar, Ward Hill, MA, USA). The concentrations of PDDA and PSS in the solutions were 0.02 M and 0.01 M, respectively [9]. Prior to PEM formation the substrates were rinsed with ethanol, acetone then ethanol again and dried under N_2 flow. The substrates were first immersed into the PDDA solution for 10 min. Upon removal from the PDDA solution the substrates were rinsed for 30 s in deionized water then dried under N_2 flow. This was repeated with the PSS solution. The PDDA–PSS sequence was repeated until a 9-layer PEM was generated with the last layer positively charged PDDA, as schematically described in Fig. 1a.

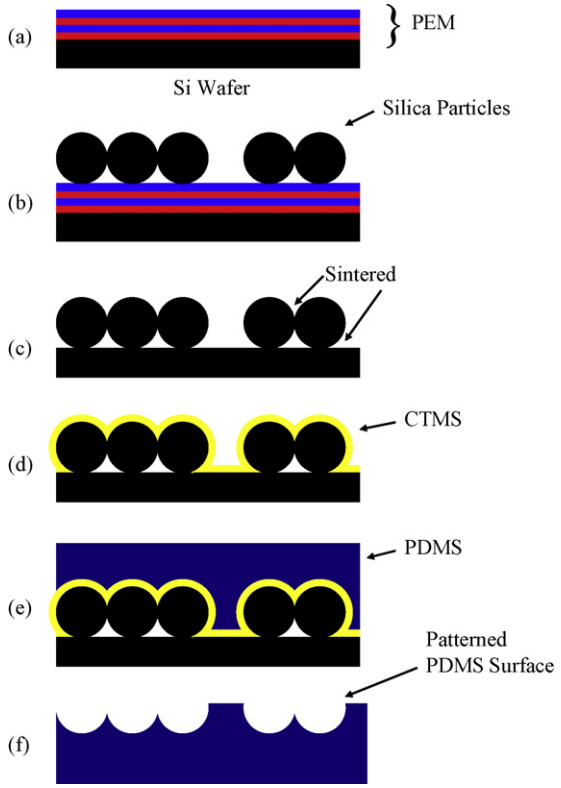

Fig. 1. Schematic of patterned PDMS fabrication sequence: (a) PEM deposition on a silicon substrate with positively charged PDDA as the last layer of the PEM structure; (b) negatively charged silica particles deposited electrostatically on the PEM surface; (c) the silica particles sintered to the silicon surface while removing the PEM by thermal decomposition; (d) deposition of CTMS as a releasing agent to help release the patterned PDMS sample from the mold surface; (e) reverse replication of the mold surface to create the patterned PDMS surface; and (f) illustration of the patterened PDMS surface with some areas that were not patterned due to the incomplete coverage of silica particles on the silicon surface as explained in the text.

2.3. Self-assembly of silica particles, sintering and application of releasing agent

After PEM formation the substrates were rinsed thoroughly with deionized water to lower the amount of NaCl present in the PEM structure. The substrates were then dipped into a colloid suspension of 0.5, 1.0 and 2.0 μm silica particles (Polysciences, Inc., Warrington, PA, USA) at a concentration of 2.5 wt.% for 10 min. The silica particles were negatively charged and therefore electrostatically attracted to the positively charged surface of the PEM. Upon removal the substrates were rinsed with deionized water to remove any unadsorbed particles beyond the first layer of particles that were electrostatically deposited on the silicon surface (Fig. 1b). The substrates were then placed in a furnace (Microtherm MT 11, The MELLEN Company, Inc., Concord, NH, USA) for sintering. The sintering step was used to increase the mechanical bonding of the silica particles to the silicon surface so that: (1) the particles would not be removed from the mold during the production of patterned PDMS surfaces and (2) the mold could be used multiple times. Several conditions were evaluated to optimize the sintering step: temperatures of 800, 900, 1000, and 1100 °C and times of 1, 5, 10, and 15 h. For each sintering condition, PDMS was poured over the mold, removed and observed under SEM to determine (1) if particles had been removed and (2) if the pattern had been successfully transferred. Through this evaluation process, 1100 °C and 15 h were selected as optimum temperature and time for the sintering step. The furnace ramping rate was 10 °C/min. This sintering step also removed the PEM from the substrate as illustrated in Fig. 1c. We have previously determined that the PEM decomposes completely around 600 °C [10]. Once sintering conditions were finalized the reusability of molds was studied. During initial testing the PDMS was difficult to remove and small traces of PDMS residue were left on the mold. A releasing agent, chlorotrimethylsilane (CTMS, >99.0%, Sigma–Aldrich, Germany), was applied to ease the removal of PDMS from the molds as illustrated in Fig. 1d and e. The molds were placed in a vacuum chamber along with 1 ml of CTMS for 3 min in order to apply the releasing agent.

2.4. Production and replication of PDMS substrate

The PDMS was produced by mixing a prepolymer (Sylgard® 184 Silicone Elastomer Base, Dow Corning, Midland, MI, USA) with a cross-linking agent (Sylgard® 184 Silicone Elastomer Curing Agent, Dow Corning, Midland, MI, USA) at a 10:1 ratio. The solution was mixed and defoamed using a Thinky Mixer (Planetary Centrifugal Mixer, ARE-250, Thinky Corporation, Tokyo, Japan) for 1 min at 2000 rpm. The mixture was poured over the silicon molds in a Petri dish using a syringe to a depth of 0.42 cm and cured for 4 h at 70 °C. After curing the excess PDMS was cut away from the mold and the patterned surface was peeled off. This was then repeated for further replications. Samples were autoclaved prior to use. A schematic of the patterned PDMS surface is shown as the final step in Fig. 1f. In addition to the patterned PDMS surfaces, a plain wafer treated with CTMS in a similar manner was used to produce plain PDMS surfaces as a control.

2.5. Inoculum preparation

Trypticase soy broth (TSB, MP Biomedicals Inc., Solon, OH, USA, 30 g/l), supplemented with yeast extract (Acros, Morris Plains, NJ, USA, 6.0 g/l) and glucose (J.T. Baker, Phillipsburg, NJ, USA, 8.0 g/l), was used as the culture medium. *S. epidermidis* strain NJ9709 [11], which was isolated from the surface of an infected intravenous catheter, was used. A colony of *S. epidermidis* was removed from the source agar plate using an inoculating loop. The colony