Patterning Bacteria within Hyperbranched Polymer Film Templates

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A four-step soft lithographic process based on microcontact printing (μCP) of organic monolayers, hyperbranched polymer grafting, and subsequent polymer functionalization, results in polymer/*n*-alkanethiol patterns that direct the seeding of bacterial cells. The functional units on these surfaces are three-dimensional bacteria "corrals" that are as small as 12 μm square. The corrals have hydrophobic, methyl-terminated *n*-alkanethiol bottoms, which promote bacterial adhesion, and walls consisting of hydrophilic poly(acrylic acid)/poly(ethylene glycol) layered nanocomposites that inhibit adhesion. Cell viability studies indicate that cells remain viable on the patterned surfaces. Large corrals (63 μm square) contain 18 ± 5 bacteria and smaller corrals (12 μm square) contain 2 ± 1 bacteria. Bacteria reside within corrals with a reliability of 92 ± 8%; the remaining cells reside on walls between corrals. Applications to bioarrays for high-throughput screening and biosensors are envisioned.

Introduction

In this paper we report a simple method for micropatterning bacterial cells. These results expand upon our earlier finding that corrals consisting of composite poly(acrylic acid)/poly(ethylene glycol) (PAA/PEG) walls and *n*-hexadecylthiol (C16SH) interiors are suitable for patterning a number of different mammalian cell lines, including macrophages, endothelial cells, and hepatocytes.[1−3] Here we show that this same micropatterning approach can be used to prepare polymeric corrals having critical dimensions as small as 12 μm. This is significant, because it permits preparation of large-scale bacterial arrays that hold promise as platforms for biosensing and high-throughput screening.

We recently showed that it is possible to fabricate hyperbranched polymer film (HPF) patterns using both microcontact printing (μCP)[1,4−6] and photoacid patterning.[1,7] In the μCP approach,[8−12] which was used for the work described here, an elastomeric stamp is used to transfer a well-defined pattern of *n*-hexadecanethiol (C16SH) to a Au-coated substrate. Next, the substrate is exposed to mercaptoundecanoic acid (MUA), which forms a monolayer on areas of the substrate not occupied by the C16SH pattern. Subsequent selective grafting of three layers of PAA followed by a layer of PEG (3-PAA/PEG) to the MUA fraction of the SAM resulted in the formation of corrals having 52 ± 2 nm high composite-polymer walls and 1.8 nm thick C16SH bottoms.[1,5,13]

A number of other methods have been reported for patterning biomaterials (mainly cells and proteins). The first class of techniques involves direct transfer of the biomaterial to a substrate using a suitably prepared master stamp. A number of specific methods within this class have been described, including: microcontact printing,[8,9,12,14−16] membrane-based patterning,[17] micromolding in capillaries,[12,18,19] and laminar flow patterning.[12,18,20] The second class of biopatterning methods relies on selective chemical or physical modification of the substrate surface to control cell adhesion. This approach has typically relied on linking of the biomaterial through a specific interaction (e.g., a covalent bond or a protein−protein interaction), or manipulation of surface charge,[21] hydrophilicity,[21] or topography.[12,14,22]

Bacterial adhesion to surfaces is not well understood, however there is general agreement on some of the basic principles involved.[23,24] When a bacterium first encounters the substrate surface it adheres reversibly due to non-

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specific interactions,[25,26] such as surface hydrophobicity, van der Waals or electrostatic forces, or acid−base interactions.[27] Once reversible attachment is made, it is possible for the bacterium to irreversibly adhere to the surface through the formation of protein−ligand bonds and the formation of an extracellular polymer.[27] Additionally, bacteria bound to surfaces have the ability to produce a "bioslime", which enables them to form colonies.[28,29] Several techniques have been used recently to better understand bacterial adhesion, including optical tweezers,[23] atomic force microscopy (AFM),[26] and total internal reflection aqueous fluorescence (TIRAF) microscopy.[24]

Most cells, including bacteria, adhere to surfaces through the formation of a protein layer. Accordingly, an important method for preventing cell adhesion is to prevent the formation of the protein layer.[30] The most common approach for accomplishing this is to add a layer of poly(ethylene glycol) (PEG) to the surface.[30−32] Although the mechanism by which PEG inhibits biofouling is not well understood,[33−36] self-assembled monolayers terminated in ethylene glycol oligomers and other functional groups are also used to prevent bioadhesion.[33,34,37−41] Protein adhesion can also be minimized by treating surfaces with phospholipids or polysaccharides.[30]

We have found that PEG grafted atop a PAA hyperbranched polymer significantly reduces bacterial adhesion. Here we show that this approach can be used to pattern bacteria with 12 μm resolution and better than 90% pattern fidelity. The advantage of using a relatively thick polymer for pattern formation is that the hyperbranched polymer fills in defects that might arise on patterns derived exclusively from monolayers. Additionally, polymer films are generally more resilient than monolayers.[1,2] Finally, the polymer films contain a high density of unreacted acid groups that could be used for further elaboration of the scaffold.

Experimental Section

Materials and Chemicals. *tert*-Butyl acrylate, 1,1′-carbonyldiimidazole, 4,4′-azobis(4-cyanovaleric acid) (75+%), ethylenediamine (99%), dichloromethane (anhydrous), 11-mercaptoundecanoic acid (MUA), hexadecanethiol (C16SH), *N*-methylmorpholine, ethyl chloroformate, *N*, *N*-dimethylformamide (anhydrous), ethyl acetate (anhydrous), methanesulfonic acid, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), ampicillin, and all buffer components were purchased from the Aldrich Chemical Co. (Milwaukee, WI). All chemicals were used as received except for the *tert*-butyl acrylate, which was distilled to remove polymerization inhibitors. Methoxy poly(ethylene glycol) amine (PEG) with a molecular weight of 5000 was purchased from Shearwater Polymers (Huntsville, AL) and used as received. Hexamethyldisilizane was purchased from Ted Pella, Inc. (Redding, CA) and used as received. LIVE/DEAD *Bac*Light Bacteria Viability Kit (L-7007) was purchased from Molecular Probes (Eugene, OR). Tryptone peptone, yeast extract, and granulated agar were purchased from Fisher Scientific (Atlanta, GA).

Microfabrication of Patterned Surfaces. Cell corrals consisting of 3-PAA/PEG walls and C16SH interiors were prepared on Au-coated Si wafers using a previously published method.[1,4−6] After patterning, the wafers were sterilized by exposure to 254 nm UV light for 2 h before seeding with bacteria. We have previously shown that this sterilization method has no negative impact on the patterned wafer.[2]

Atomic Force Microscopy. Tapping mode atomic force microscopy (TM-AFM) images were obtained using a Digital Instruments Nanoscope III (Santa Barbara, CA) scanning probe microscope fitted with a *j* scanner (200 μm range). The TM-AFM cantilevers were purchased from NanoSensors (Wetzlar-Blankenfeld, Germany). These cantilevers had resonance frequencies between 260 and 280 kHz, force constants between 20 and 100 N/m, and tip apex radii of approximately 10 nm. Images were acquired at 512 × 512 pixels with a 0.1−0.5 Hz and near-minimal contact force.

Cell Culture Conditions. Agar plates containing 15 g/L of granulated agar and a liquid broth solution were prepared. The liquid broth solution contained 10 g/L of tryptone, 5 g/L yeast extract, and 10 g/L of NaCl. The liquid broth was heat sterilized by steam autoclaving at 120 °C for 15 min. 100 mg/mL of ampicillin was added from a stock solution that had been sterilized using a 0.2 μm syringe filter. Ampicillin inhibits the growth of bacteria that are not ampicillin resistant. The agar was poured into disposable polystyrene Petri dished and allowed to cool to room temperature until the agar was fully congealed, and then they were stored at 4 °C until needed. Bacteria (BL21 *E. coli* from Novagene) were streaked on an agar plate and incubated at 37 °C for a minimum of 12 h. These preparations could be stored for up to 4 weeks at 4 °C. All work involving bacteria was performed using sterile equipment.

Bacteria were removed from the agar plate by scraping off a colony with a sterile micropipet tip. The tip was then placed in 10 mL of liquid broth and incubated at 37 °C overnight. The bacteria were then centrifuged and resuspended in 0.1 M phosphate buffer (0.144 g/L KH_2PO_4, 9.00 g/L NaCl, and 0.795 g/L $Na_2HPO_4 \cdot 7H_2O$ adjusted to pH 7.4 using 1 M NaOH or 1 M HCl; 100 μg/L of ampicillin was added and the buffer was sterilized by passing it through a 0.2 μm bottle-top filter) to yield an approximate cell concentration of 1 mg/mL (1×10^9 cells/mL). The polymeric patterns (corrals) were seeded with bacteria by placing the patterned wafer inside a flow cell constructed from poly(dimethylsiloxane) (PDMS). The solution containing the bacteria suspended in the buffer was passed through the flow cell, and then the flow was stopped for 15 min. The wafer was then rinsed gently using PBS.

Cell Viability Assays. The LIVE/DEAD *Bac*Light Bacteria Viability Kit was used to examine the bacteria on the patterned wafer. The kit contains a combination of two nucleic acid stains; SYTO 9 and propidium iodide. SYTO 9 fluoresces green and labels all of the bacteria and the propidium iodide fluoresces red and only labels bacteria with compromised membranes. The result is that live bacteria fluoresce green and dead bacteria fluoresce red. To determine if the bacteria on the patterned substrates were alive or dead, the wafer was incubated for 15 min in a solution containing 2 mL of each dye component per 1 mL of HEPES Buffered Saline Solution (HBSS) in the dark. HBSS was prepared using 7.89 g/L NaCl, 0.38 g/L KCl, 0.10 g/L $MgSO_4$, 0.20 g/L $CaCl_2$, and 2.38 g/L HEPES. The pH of the solution was

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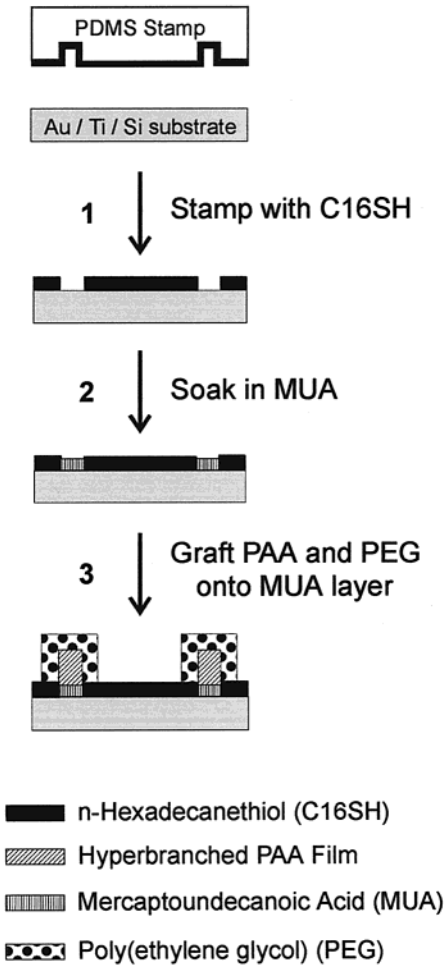
Scheme 1



adjusted to 7.4 using 1 M NaOH or 1 M HCl and filtered using a 0.2 μm bottle-top filter. The wafer was then rinsed with HBSS and soaked in 4% gluteraldehyde solution for a minimum of 15 min in the dark and rinsed with Milli-Q water before it is dried by gently blowing N_2 over the wafer. The wafer was then imaged using a FITC filter set on a Nikon Eclipse TE300 Inverted Fluorescence Microscope (Melville, NY) using a Nikon N2000 camera and Fujicolor Superia 800 speed film.

Results and Discussion

Characterization of Microfabricated Patterns. We have previously shown that it is possible to pattern mammalian cells using PEG-capped hyperbranched polymer films.[2,3] The lithographic approach for fabricating corrals for bacterial patterning is shown in Scheme 1. First, a pattern of C16SH is transferred from an elastomeric PDMS stamp to a clean Au substrate to define the bottom of the corrals. Second, the wafer is immersed in a MUA solution to yield a monolayer having a reactive acid terminal group on regions of the surface not previously passivated by C16SH. Third, the walls of the corrals are formed by selectively grafting three layers of PAA onto the MUA regions of the pattern.[1,4,6] To reduce bioadhesion on the walls, the hyperbranched polymer is capped with a layer of PEG to yield the final 3-PAA/PEG structured nanocomposite.

The patterns prepared according to Scheme 1 were characterized by ellipsometry, Fourier transform infrared-external reflection spectroscopy (FTIR-ERS), tapping made-atomic force microscopy (TM-AFM), and X-ray photoelectron spectroscopy (XPS).[2,3,6] The chemical and physical characteristics of the corrals were found to be consistent with those we have reported previously.[1]

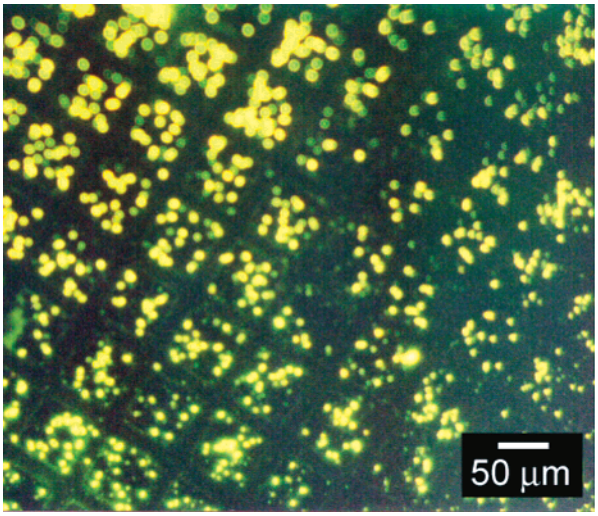
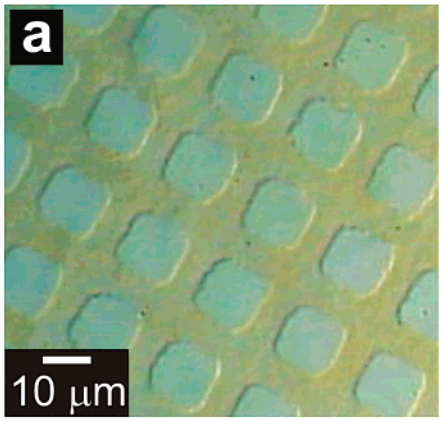


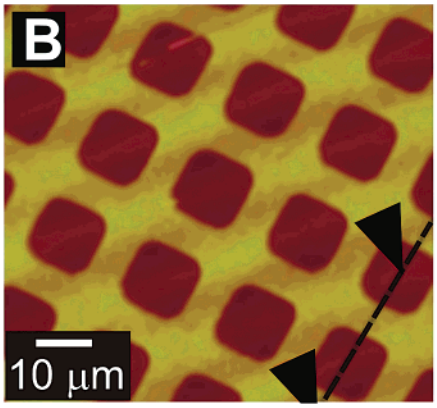
Figure 1. Fluorescence micrograph of bacteria patterned within an array of corrals. The corral interiors are 63 μm square and have bottoms consisting of a self-assembled monolayer of C16SH. The walls are 52 ± 2 nm high and consist of a 3-PAA/PEG structured nanocomposite. The *E. coli*. were stained with Molecular Probes LIVE/DEAD assay kit; the green fluorescence indicates that the cells are viable.

The relative sizes of the host corral and the guest cell determine the maximum number of cells that each corral can contain. For example, we recently described the use of 63 μm square polymeric corrals for patterning mammalian cells.[1−3,6] Mammalian cells normally range in size from 10 to 100 μm in diameter, and therefore each corral typically contains 1−5 cells.[1] In contrast, the fluorescence micrograph shown in Figure 1 reveals that ∼18 ± 5 bacteria (which are typically about an order of magnitude smaller than mammalian cells) occupy corrals of this size. As discussed in the Experimental Section, the patterned cells were treated with a live/dead assay kit; the green fluorescence observed from these patterned bacteria indicate that they are viable on the patterned substrate.

Because we plan to use arrays of cells as sensor elements, we endeavor to construct corrals that will hold one or just a few bacteria. Figure 2a shows an optical image of an array of corrals having interior dimensions of ∼12 μm. These features were patterned using a 1500-mesh TEM grid as the master for the PDMS stamp. The features of the corral are very similar to those of the 63 μm square corrals. The micrograph indicates that the corral interiors are 12 μm on a side and that the walls are ∼5 μm wide. The AFM image shown in Figure 2b, along with the line scan in Figure 2c, confirm the data obtained from the optical micrograph. Additionally, they indicate that the corral walls have a uniform height and width: 30 nm and 5 μm, respectively. In contrast, the array consisting of 63 μm wide corrals had walls that were nearly twice as high even though they were synthesized in the same manner. The significantly lower value here may be related to the reduction in scale of the hyperbranched polymer chemistry.[1,2]

Figure 3 shows a fluorescence micrograph of *E. coli* cells seeded onto a pattern similar to that shown in Figure 2. There are two important conclusions that can be drawn from this micrograph. First, the observation of green fluorescence from the bacteria indicates that the cells are viable on this smaller grid. Second, the smaller size of the corrals results in a nearly 10-fold reduction in the number of bacteria per corral compared to the larger features





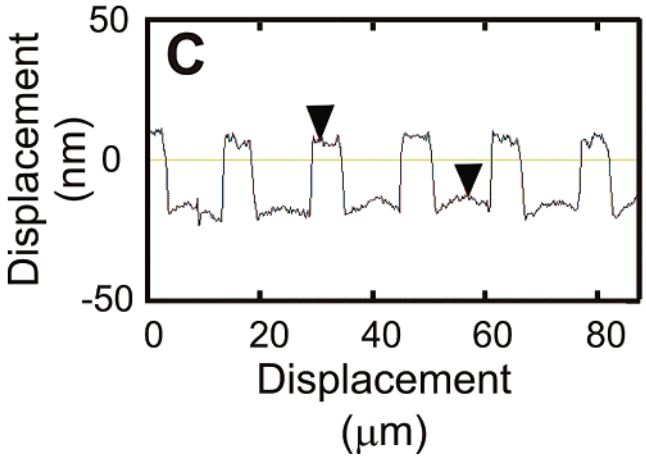


Figure 2. (a) Optical and (b) tapping-mode atomic force micrographs of a pattern consisting of corrals having interior dimensions of 12 μm. The walls consist of a 3-PAA/PEG structured nanocomposite. The AFM line scan in (c) indicates that the walls are geometrically uniform and have widths of 5 μm and heights of 30 nm. The pattern in (a) was stained with crystal violet to provide image contrast.

shown in Figure 1: 18 ± 5 vs 2 ± 1 bacteria/corral (for the 12 μm pattern, corrals not containing bacteria were not included in these figures). A key aspect associated with placing cells on smaller patterns is that there is a higher ratio of wall space compared to interior corral. All other

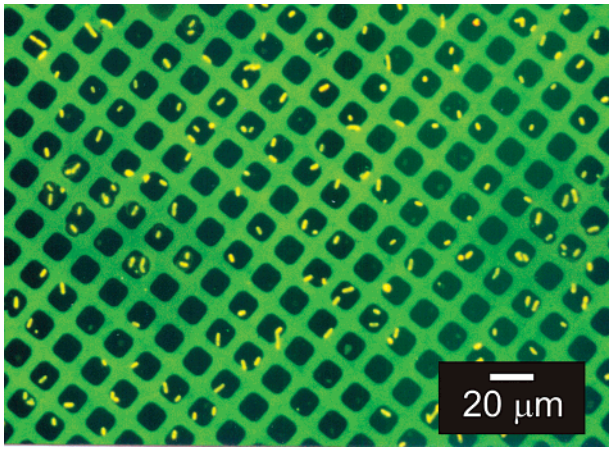


Figure 3. A corral array prepared identically to that shown in Figure 2 after seeding with *E. coli*. The presence of green fluorescence indicates that the bacteria are viable on the patterned surface. Not including the empty corrals, there are 2 ± 1 bacteria per corral.

parameters being equal, this means there is a higher likelihood that errors in cell placement (that is, cells on walls) will occur. However, on the basis of 10 randomly chosen regions on three independently prepared patterned wafers, we found that an average of $92 \pm 8\%$ of the cells were in the corrals rather than on the walls. Note, however, that if bacteria are allowed to grow on the patterns for 6−12 h, they generate a bioslime that permits a much higher percentage to reside outside of the coral boundaries.

Conclusions

We have previously shown that an array of hyperbranched polymer corrals can be fabricated by microcontact printing followed by iterative polymer grafting, hydrolysis, and activation steps.[1] When PEG is grafted atop the walls of such patterns, adsorption of both proteins and mammalian cells is inhibited. This results in the spatial confinement of cell growth to the corrals. Here, this finding was extended to show that much smaller corrals can be micropatterned, and that these micropatterns template bacterial adhesion with good (but not perfect) fidelity. Bacteria residing within these corrals were found to be viable.

The ability to pattern small numbers of bacteria within polymeric patterns opens the door to a wide range of potential applications. For example, it should be possible to prepare large libraries of mutant bacteria, seed them into individual corrals, and then individually examine each biopixel in the array when they are dosed with small molecules such as drug candidates or environmental analytes. Microfluidic systems should be ideally suited for implementing such assays on bioarrays such as these.

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