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Patterning and Separating Infected Bacteria Using Host–Parasite and Virus–Antibody Interactions

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Abstract. **Bacteria were selectively deposited on substrates patterned with poly(ethylene glycol) (PEG) microstructures by using host–parasite and virus–antibody interactions. In this scheme viruses were used to attach onto a host bacterium, Escherichia coli (*E. coli*). The *E. coli* expressing the virus were selectively adhered to the regions pretreated with an antibody against the virus proteins while *E. coli* without the virus showed no selectivity. Single or aggregated cell arrays were fabricated depending on the initial pattern size with respect to the size of *E. coli*. The current approach could be a general route to spatially positioning or controlling adhesion of other biological species that are not accessible by conventional methods and as a tool for separating and isolating specific cell populations based on host–parasite interactions.**

Key Words. **poly(ethylene glycol) microstructures, host–parasite interactions, capillary lithography, bacteria, patterning**

1. Introduction

Miniaturization in cell-based bioassays is currently under active study for the screening of large libraries of potential pharmaceutical agents or the detection of toxic compounds and pathogens in the environment (Dunn and Feygin, 2000). In particular, bacterial cells are strong candidates for sensing applications since analytic specificity can be readily modified by genetic engineering and these microorganisms are relatively robust as compared to mammalian cells (Belkin et al., 1997; Larsen et al., 1997; Liao et al., 2001; Rainina et al., 1996). Thus, the ability to selectively attach bacterial cells to micropatterned surfaces is potentially of benefit to create sensor arrays for rapid screening of infectious diseases or detecting toxic compounds (Cowan et al., 2001; Ilic et al., 2000).

To enable the fabrication of micropatterned surfaces, the nature and mechanism of bacterial adhesion must be understood in depth (Liang et al., 2000; Vigeant et al., 2001). Generally, when a bacterium first encounters the substrate surface, it adheres reversibly due to non-specific interactions (Bonin et al., 2001; Ong et al., 1999) and subsequently it adheres irreversibly through the formation of protein-ligand interactions (Razatos et al., 1998). Nonetheless, the adhesion must be aided by the formation of a protein layer, which is valid for most other cell types as well. Therefore, a protein-resistant layer has been used to prevent both non-specific adhesion of the bacteria and adsorption of proteins onto the surface. Such barrier layers include poly(ethylene glycol) (PEG) polymers, self-assembled monolayers (SAMs) terminated with a PEG group, phospholipids or polysaccharides (Kingshott and Griesser, 1999).

Based on this idea, a number of methods have been employed for patterning bacterial cells by spatially positioning or controlling bacterial adhesion on solid surfaces. These techniques include photolithography (Koh et al., 2003b), microcontact printing (μCP) (Howell et al., 2003; Ostuni et al., 2001; Rowan et al., 2002) and microfluidic channels (Cabrera and Yager, 2001; Gomez et al., 2002; Koh et al., 2003a). In particular, μCP can be used in two ways; (i) SAMs that are resistant to bacterial adhesion were grafted to surfaces to provide ''inert'' properties (Ostuni et al., 2001; Rowan et al., 2002) or (ii) patterning antibodies into a microarray was performed and subsequently the array was used to detect or immobilize a bacterium that has specific interactions with the antibody (Howell et al.,

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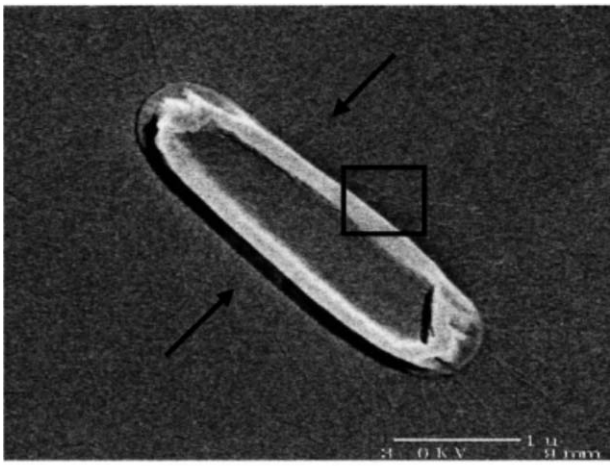
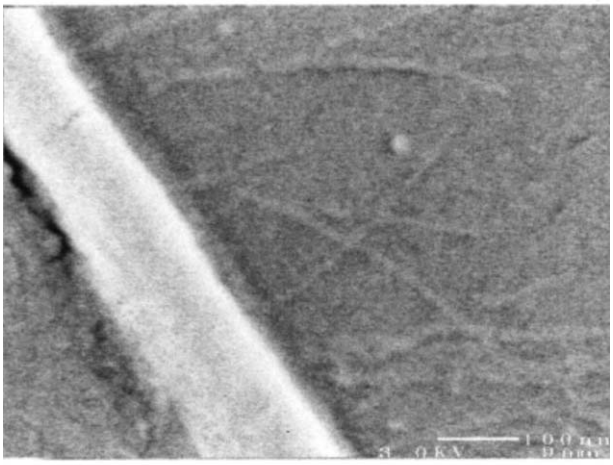
2003). Photolithography has also been used in fabricating bacterial arrays within PEG microstructures on silicon, glass, or poly(dimethylsiloxane) (PDMS) surfaces (Koh et al., 2003b).

Here we present an alternative method to pattern bacterial cells by utilizing host–parasite and virus–antibody interactions. In this scheme, in addition to using a PEG copolymer as a protein-resistant layer, virus-antibody interactions were introduced to enhance the selectivity. Our preliminary experiments demonstrate that the adhesion of bacteria was significantly reduced on PEG surfaces compared to bare silicon or glass substrates. However, non-specific adhesion became noticeable as the concentration of bacteria increased, leading to an undesirable reduction in selectivity. More importantly, the current approach would provide routes to cell separation/isolation or purification since host–parasite interactions are naturally occurring phenomena in the tissue. Once viruses were attached onto a host bacterium to interact with the specific antibody on the surface, cells that have been infected could be separated from non-infected cells and then detected for further analysis. Host–parasite interactions are relatively well-established and a number of parasites are available for a given host. Thus, this approach could be applied to other host-parasite combinations and could potentially be used for detecting the presence of host contaminations or purifying specific cell populations.

To allow for physical microstructures for confining ''infected'' bacteria, we used capillary lithography that was reported earlier (Kim et al., 2001; Suh et al., 2001). Capillary lithography is a simple molding technique to provide polymeric microstructures virtually on any surface. Recently, we applied the technique to various biomaterials such as hyaluronic acid (HA) (Khademhosseini et al., 2004b; Suh et al., 2004a) and PEGs (Khademhosseini et al., 2003; Suh et al., 2004b) as an ink/medium for creating protein or cell arrays without involving any chemical modifications. In addition, the technique was also applied to fabricating microstructures inside microfluidic channels for protein or cellular arrays (Khademhosseini et al., 2004a). The results presented here expand upon our earlier findings, with major focus on smaller biological species using smaller microstructures. For the patterning, relatively thick PEG microstructures were produced using capillary lithography for templating the adhesion of bacteria. It is noted that PEG microstructures have a number of advantages over SAMs including (i) less defects, (ii) more resiliency, and (iii) higher density. M13 virus and *E. coli* were used as a parasite and a host, respectively. In principle, however, our method is not limited to a specific virus or bacterium as long as a certain antibody is available to interact with the corresponding virus.



(a)



(b)

Fig. 1. *(a) A SEM micrograph of an attached E. coli to a silicon substrate that was cultured with the virus; (b) a magnified view of the boxed region in (a).*

2. Materials and Methods

2.1. Materials

A PEG-based random copolymer, poly(3-trimethoxysilyl)propyl methacrylate-r-polyethylene glycol methyl ether) [poly(TMSMA-r-PEGMA)] was used in the experiments. Details on properties and characterizations of this polymer are described elsewhere (Jon et al., 2003).

2.2. Escherichia coli (E. coli) and M13 culture

The M13 phage display containing the M13 phage and *E. coli* was obtained from New England Biolabs (Beverly, MA). The *E. coli* was grown in Erlenmeyer flasks that were filled with 20 mL LB medium (10 g/L Bacto-Tryptone, 5 g yeast extract, 5 g NaCl) and shaken

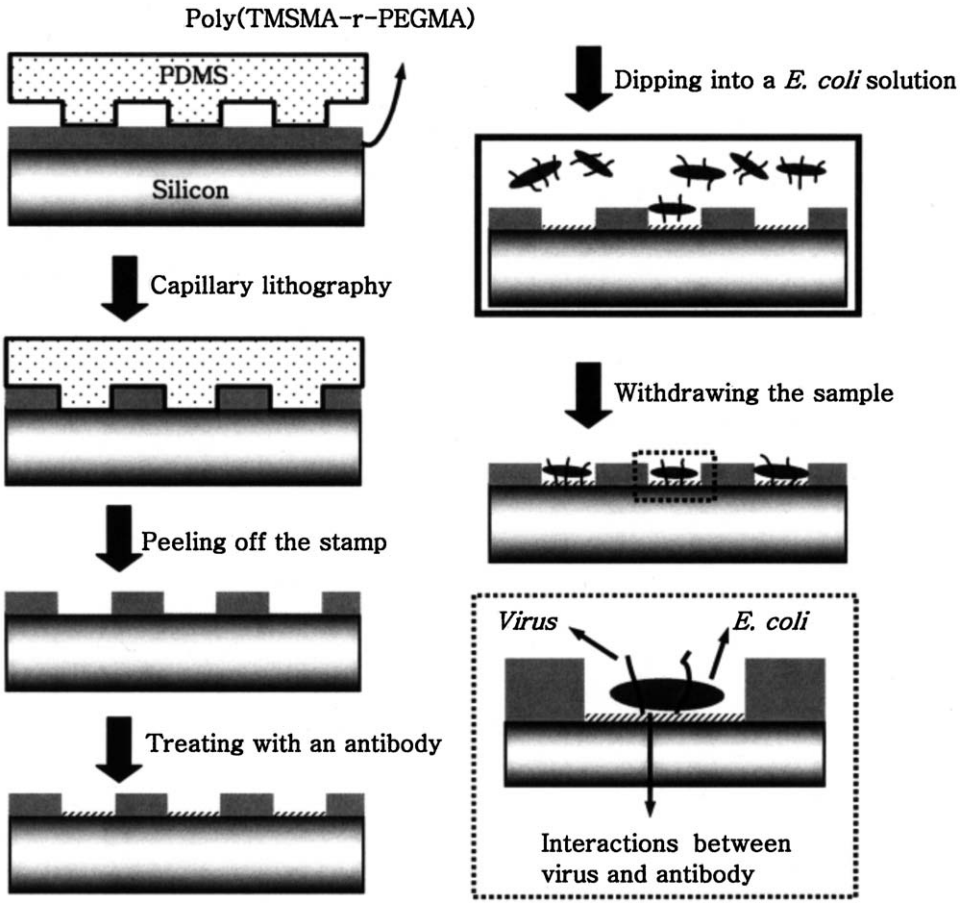


Fig. 2. *Experimental procedure of fabricating bacteria arrays using capillary lithography and host-parasite/virus-antibody interactions.*

continuously at 37 °C. To maintain the cultures, cells were diluted at 1 : 100 in fresh LB medium every 24 hours. To prepare the M13 phage, the *E. coli* were grown in an overnight culture of 1 : 100 in 20 mL LB medium. Subsequently, a solution con-taining $\sim 1.5 \times 10^{11}$ virions was added to the mixture and shaken vigorously for 4.5 hours at 37 °C. This allowed enough time for virions to adhere to the bacteria.

2.3. Fabrication of PDMS stamps

PDMS stamps were fabricated by casting PDMS (Sylgard 184 elastomer, Essex Chemical) against silicon masters prepared by photolithography (1 : 10 ratio of the curing agent). Then the pre-polymer was well mixed and incubated at 75 °C for 1 hour. After curing, PDMS stamps were detached from the master and cut prior to use.

2.4. Capillary lithography

A few drops of a 1 to 10 wt% solution of poly(TMSMA-r-PEGMA) solution were placed on a silicon wafer and a thin film of the copolymer was obtained by spin coating (Model CB 15, Headaway Research, Inc.) at 1,000 rpm for 10 seconds. To make conformal contact, PDMS stamps were carefully placed onto the surface and the samples were stored overnight at room temperature to allow for evaporation of the solvent. The film thickness after solvent evaporation ranges from 32 nm to 370 nm as determined by ellipsometry (Gaertner L116A, Gaertner Scientific Corp.) and atomic force microscopy (AFM).

2.5. Patterning M13 and proteins on the surface

To pattern the antibody on the surface, the P3 (Sigma-Aldrich Co., MO) and P9 (New England Biolabs, MA) antibodies were diluted at 1 : 100 in PBS. A few drops of the antibody solution were then evenly spread on the patterned surface for 1 hour. The resulting patterns were either stained with secondary antibodies to determine protein distribution or treated with a solution of M13. To pattern the *E. coli* on the surfaces, the patterns were placed within a dish containing the *E. coli* containing solution for 4 hours, prior to analysis.

2.6. Atomic force microscopy (AFM)

AFM images were taken in tapping mode on a NanoScope III Dimension (Veeco Instruments Inc.) in

air. The scan rate was 0.5 Hz and 256 lines were scanned per sample. Tapping mode tips, NSC15—300 kHz, were obtained from MikroMasch (Portland). Data were processed using Nanoscope III 4.31r6 software (Veeco Instruments Inc.). Some of the images shown were flattened but not further manipulated.

2.7. Scanning electron microscopy (SEM)

Virus images were taken using high-resolution SEM (JEOL 6320FV, MIT) at an acceleration voltage of 3 eV and a working distance of 7 mm. Sample were coated with a 15 nm Au layer prior to analysis to prevent charging.

3. Results and Discussion

3.1. E. coli culture with M13 virus and attachment to a surface

The M13 virus has an average length of $\sim$ 900 nm and a width of $\sim$ 6 nm. A typical result is shown in Figure 1 for an attached *E. coli* cultured with the virus on a silicon surface that was pretreated with the P3 antibody. The antibody was known to specifically interact with a core protein in the virus (P3 attaches to the proteins on the long edges of the virus while P9 attaches to the end. For most experiments, P3 was used for its better performance). No such interactions are expected between *E. coli* and the antibody. The arrows in Figure 1a indicate numerous viruses spreading from the *E. coli* to the surface. Figure 1b shows the zoomed region in Figure 1a, in which the viruses acted as an adhesive in immobilizing the *E. coli*.

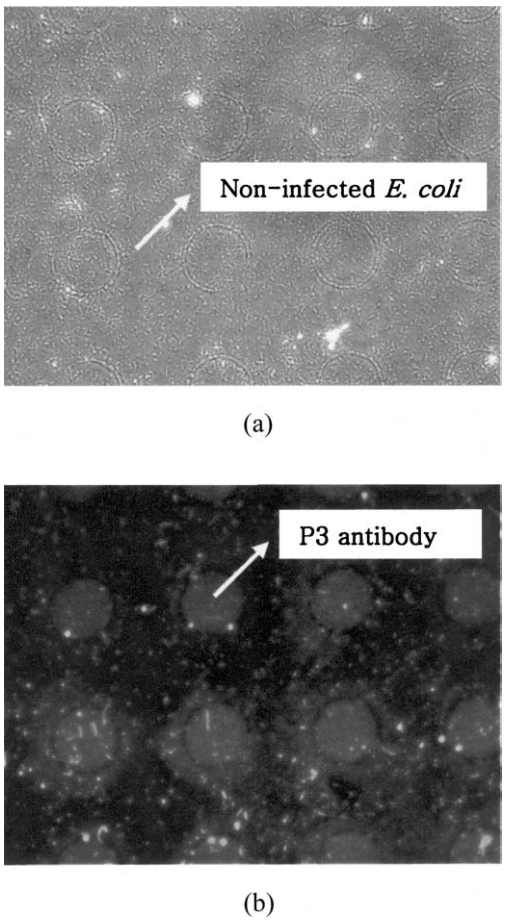


Fig. 3. *(a) An optical micrograph for patterning E. coli onto 50 μm circles in the absence of virus. No selectivity in bacterial adhesion was observed; (b) a fluorescent micrograph for (a). Note that the exposed circles were treated with the antibody prior to exposure to E. coli.*

3.2. Capillary lithography and verification of non-specific interactions between E. coli and antibody

To construct microscale patterns, we used capillary lithography (Figure 2) (Kim et al., 2001; Suh et al., 2001). In this technique, the feature height can be easily controlled depending on the geometry of the stamp and film thickness. Moreover, the microstructures are intrinsically dense and robust without many defects. A potential concern with this technique is whether the substrate surface can be completely exposed, which is a prerequisite to enable the successful attachment of biological species. Recently, we found that the surface can be easily exposed with PEGs as molded polymers (Suh and Langer, 2003). In comparison to hydrophobic polymers such as polystyrene, the polymers were neatly patterned upon conformal contact with a patterned PDMS stamp, resulting in a negative replica without dewetting. In the experiment, poly(TMSMA-r-PEGMA) was used as a molded polymer. We reported earlier that this polymer forms polymeric monolayers or microstructures with simple modifications to the fabrication procedure, generating features varying in height from a few nanometers to a few hundred nanometers (Khademhosseini et al., 2003). Furthermore, both microwells and polymeric monolayers are remarkably stable in water for a period of at least two weeks.

To examine the possibility of specific interactions between *E. coli* and antibody, we fabricated 50 μm circles and treated the surface with the antibody followed by *E. coli*. As shown in Figure 3a, no selectivity in bacterial adhesion was observed between the exposed and the PEG covered regions with a number density of about $0.02\,\mu m^{-2}$. In addition, the fluorescent image in Figure 3b illustrates the nonspecific staining of DAPI on the exposed circular substrates. Based on this data it can be seen that the interactions between *E. coli* and the antibodies against viruses are negligible.

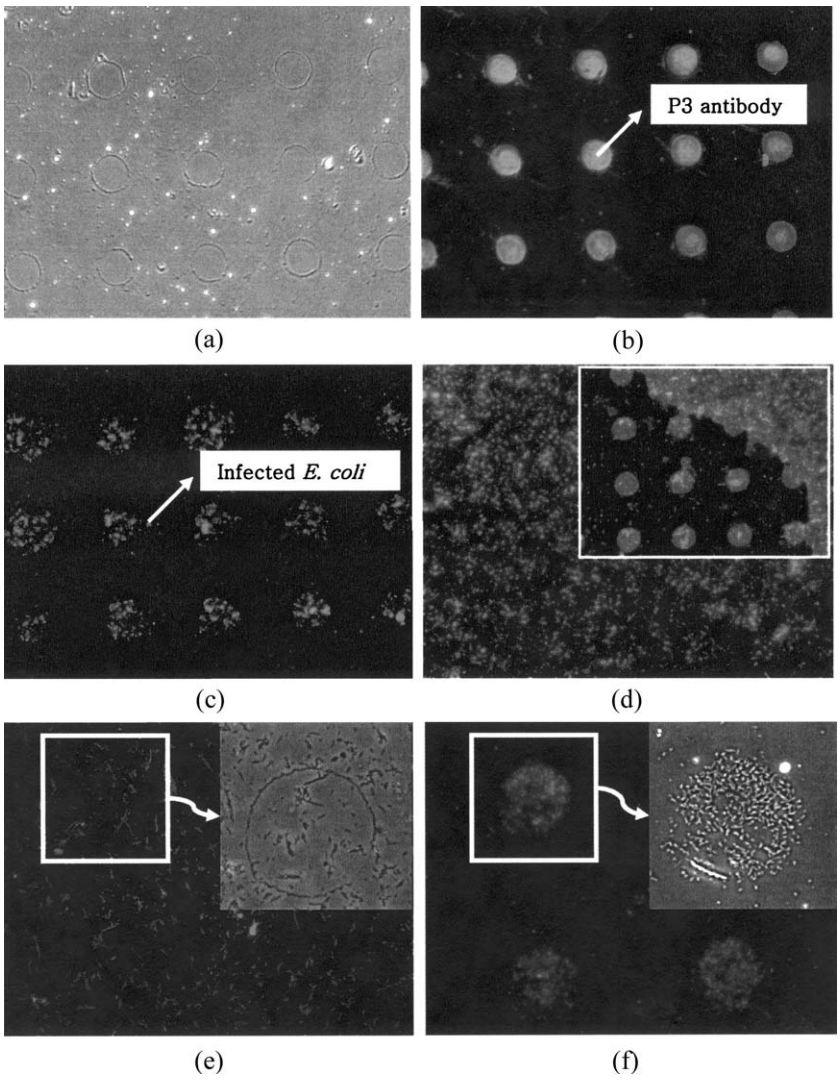


Fig. 4. *(a) An optical micrograph for the initial microstructure of 50 μm circles; (b) a fluorescent micrograph for (a) stained with a FITC-labeled secondary antibody; (c) an optical micrograph for aggregated arrays of E. coli onto the same pattern; (d) a fluorescent image for E. coli adsorption onto a bare silicon substrate without a predefined pattern (control) the inset shows the boundary between the patterned and non-patterned regions; (e) a fluorescent image for bacteria patterning without the aid of virus (inset: the corresponding optical image for the box region); and (f) a fluorescent image for the pattern stained with a different dye (inset: the corresponding optical image for the box region).*

3.3. Aggregated arrays of E. coli

To test the ability of surfaces to capture infected bacteria (i.e., bacteria that contains virions on its surface), *E. coli* were cultured with the M13 virus and then seeded onto the patterns of the PEG polymer as shown in Figure 4. For pattern generation, the initial microstructure and the antibody array should be prepared (Figures 4a and b). With the aid of the virus–antibody interactions, well-defined aggregated arrays of *E. coli* were obtained (Figure 4c). In contrast, no arrays were observed on a bare silicon substrate without a predefined PEG pattern (Figure 4d). It appears that the adhesion of bacteria was significantly enhanced on the bare silicon substrate as compared to PEG surfaces. Again, selectivity was examined in Figures 4e and f as to whether the virus affects the bacterial adhesion, which turned out to be valid for all the experiments tested.

3.4. Single arrays of E. coli

Single arrays were also tested using a small pattern, the size of which is comparable to that of *E. coli*. A PDMS stamp that has 800 nm lines with a spacing of 1 and 2 μm was used for the purpose. As shown in Figures 5a and b, the initial microstructures were neatly fabricated, with a height of about 367 nm. Seeding of *E. coli* onto such a microstructure gives rise to single arrays of *E. coli* along

the channel direction ($\sim 0.025\,\mu m^{-2}$) (Figures 5c to e). It is noted that each *E. coli* is tightly confined within the channel since the size of the channel is about the same with that of *E. coli* ($\sim$ 1 μm) (Figures 5c and e). When the channel size is increased to 2 μm, one or two bacteria can be confined for each channel and rotational freedom is allowed (Figure 5d). A careful examination of the attached *E. coli* indicates that the virus indeed acted as an adhesive for the immobilization as shown in Figure 5f.

The single arrays of *E. coli* as shown above are not readily accessible by other methods. In photolithography involving PEG polymers, a pattern size less than 10 μm might be difficult to realize since conventional masks often fail to fabricate these features and expensive equipment is needed to meet pattern fidelity. The stability of PEG hydrogels would also be challenging to overcome at this small size. In addition, μCP has shortcomings for fabricating single arrays since it cannot provide physical microstructures (Howell et al., 2003; Ostuni et al., 2001; Rowan et al., 2002). Although not shown, it was observed that the height of microstructures played an important role in creating single arrays so that a lower microstructure often fails to confine *E. coli* within the channel. As the current approach is flexible in controlling topographical heights, it could open possibilities for creating arrays of small biological species such as bacteria in a relatively simple manner.

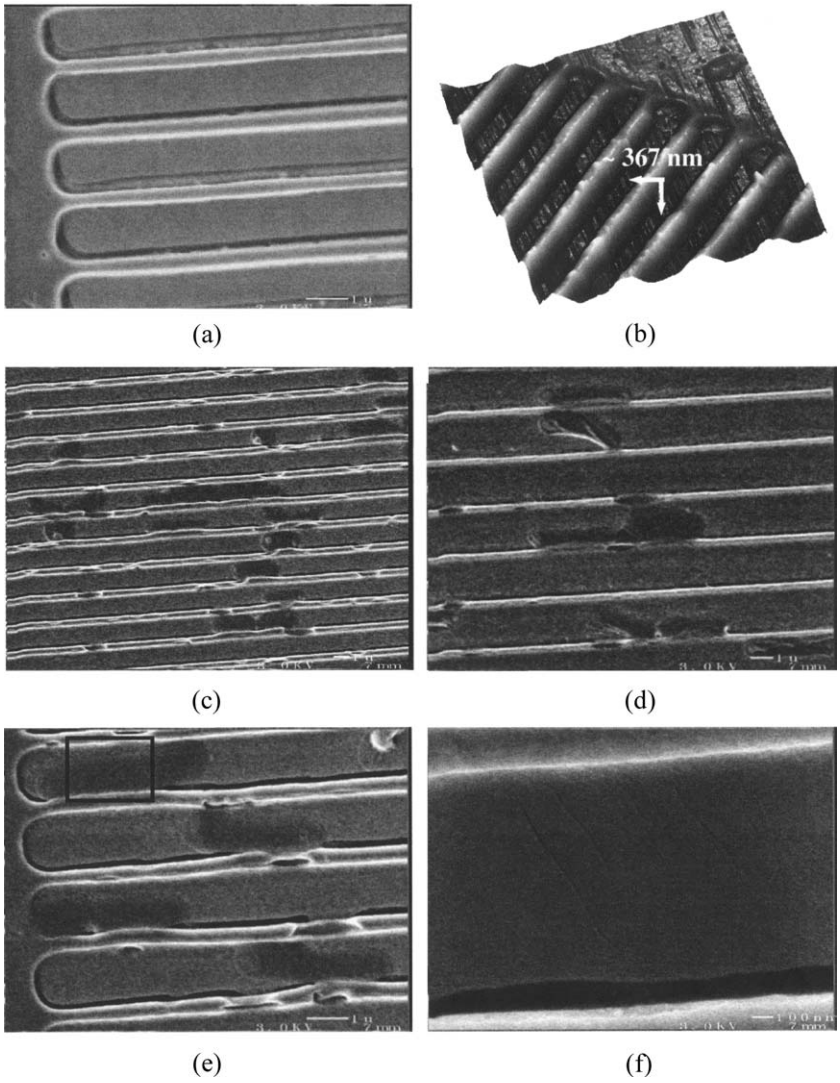


Fig. 5. *(a) An SEM image for the initial microstructure of 800 nm lines with 1 μm spacing; (b) an AFM image for (b). The scan size is $10 \times 10\,\mu m^2$. SEM images for single arrays of E. coli along the channel direction in case of (c) 1 μm and (d) 2 μm spacing. (e) An SEM image for single arrays (magnified view); (f) an SEM image for the boxed region in (e).*

4. Conclusion

In this paper, we have introduced the concept of using host-parasite and virus-antibody interactions as a tool for patterning or separating infected bacteria and demonstrated the ability to confine aggregated or single arrays of *E. coli* on silicon substrates. The infected *E. coli* were selectively adhered onto the exposed regions due to specific interactions between the embedded virus and the antibody whereas the *E. coli* without the virus showed no selectivity. Although M13 virus was used to demonstrate the basic concept, a wide range of parasites can be potentially employed depending on characteristics of the system. We believe the method could be useful in detecting the presence of bacterial contaminants or templating/stacking micro or nanostructures using bacteria. We further foresee that this concept, i.e., the use of host-parasite interactions, could be potentially of benefit in fabricating arrays of other biological species that are not easily assessable by conventional methods.

Acknowledgments

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