Polypeptide-Mediated Switchable Microarray of Bacteria

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ABSTRACT This paper describes a feasible solution for the bacterial cell death and contamination from cell division that occurs in microfluidic applications. The method adopts a smart thermoresponsive surface, highly resolved micropatterns, and surface-functionalized bacteria tagged with thermoresponsive molecules. We developed a method for controllable bacterial attachment and detachment using an elastin-like polypeptide (ELP). To create a smart surface with switchable properties, the surface of a glass substrate was conjugated with thermoresponsive ELP molecules. The attachment of bacterial cells to the ELP surface was induced by the hydrophobic affinity of the ELPs on the glass surface to tagged ELPs on the bacterial surface. A cell-repellent polymer was micropatterned to create a highly resolved space for specific bacterial adhesion. Reversible bacterial attachment and detachment was achieved by controlling the thermoresponsive phase transition of ELP molecules. Five different types of bacteria were successfully conjugated with ELPs and arrayed on the surface. The viability of the bacteria that had attached to the surface was evaluated by determining colony forming units of released bacteria on an agar plate.

KEYWORDS: bacterial microarray • thermoresponsive polypeptide • phase transition • micropattern

INTRODUCTION

Recently, bacteria have attracted considerable interest in microfluidic applications, including detectors (1), sensors (2, 3), mixers or pumps (4, 5), motors (6), and transport or delivery systems (7−9). This interest is due to several unique characteristics of bacteria. First, bacteria require only small amounts of simple nutrients such as glucose to survive. Second, the cultivation and purification of bacteria are simple, easy, and low-cost. Third, bacteria have special tactic responses to chemical attractants and repellants (2), oxygen (10), temperature differences (11), light (7), and magnetic field (12). Finally, bacterial behavior can be modified (13), and additional sensing receptor proteins can be introduced into bacteria through genetic modification (14).

For such use of bacteria in microfluidic systems, modification of the bacterial surface is required. Generally, bacterial surface chemistry shows a large charge nonuniformity and polarity caused by the localization of peptidoglycans, polysaccharides, proteins, and phospholipids. The complex nature of bacterial surfaces can lead to bacterial adhesion to many different surfaces through specific or nonspecific interactions such as electrostatic attraction (15), protein adsorption (16), surface interactions (13), nonspecific adhesion (17), and cross-linking agents (18). However, these attachment methods are mediated by irreversible interactions, and microfluidic systems that use these methods are appropriate only for single uses. In addition, microfluidic systems are easily contaminated by bacterial death or bacterial cell division, interfering with their essential functions. Most of all, the rapid loss of bacterial viability and activity on the confined surface becomes a limitation in such applications (19, 20). The switchable surface demonstrated in this paper can provide an easy way to immobilize bacteria by molecular interactions between tagged molecules on the bacterial surface and capturing molecules on the substrate. The unique feature of the process is that the immobilization of the bacteria is completely reversible and easily triggered by temperature changes without significant decreases in cell viability.

Elastin-like polypeptide(ELP), which contains repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly (VPGXG, where Xaa is any amino acid except Pro), has been identified as a smart biopolymer, as it undergoes a reversible phase transition in response to changes in temperature, pH, light, and ionic strength at its lower critical solution temperature (LCST) (21−23). ELP becomes hydrophilic and soluble in any aqueous solution below its LCST, but becomes hydrophobic and forms aggregates above its LCST (22, 23). These smart properties, as well as the biocompatibility of ELP, have facilitated its application in the fields of drug delivery, tissue engineering, and protein purification (24−26).

In this report, ELP was conjugated to the surface of bacteria and to a glass substrate. The attachment and detachment between the ELP-bacteria conjugate and the ELP-functionalized substrate were controlled by the temperature-triggered phase transition of ELP molecules. In addition, a cell-repellent polymer template was used to create a

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Received for review April 6, 2009 and accepted June 4, 2009
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DOI: 10.1021/am9002364

micropatterned surface and prevent the nonspecific adhesion of bacteria.

MATERIALS AND METHODS

Construction and Characterization of ELP. Polymerase chain reaction (PCR) and recursive directional ligation (RDL) (27) were performed to synthesize ELP with various repeating peptide units. ELP was expressed in the *Escherichia coli* strain BLR(DE3) (Novagen) with a modified pET-32b(+) vector (Novagen). All cultivation steps were carried out at 37 °C in CircleGrow medium (Qbiogene) supplemented with 100 μg/mL ampicillin. After 20 h of cultivation, the cells were harvested and resuspended in PBS (pH 7.4) buffer. The resuspended cells were lysed by sonication, and cell debris was removed by centrifugation. ELP was further purified by a repetitive phase transition followed by ultracentrifugation as described previously (27). The transition temperature (T_t) of the purified ELP was characterized by monitoring the absorbance at 350 nm using a UV spectrophotometer. 100 μM of ELP was dissolved in PBS buffer (pH 7.4) and the temperature was controlled by circulating 25−40 °C water through the holder at a rate of 1 °C/min, and the T_t was determined as the solution temperature at the half-maximum of the turbidity gradient.

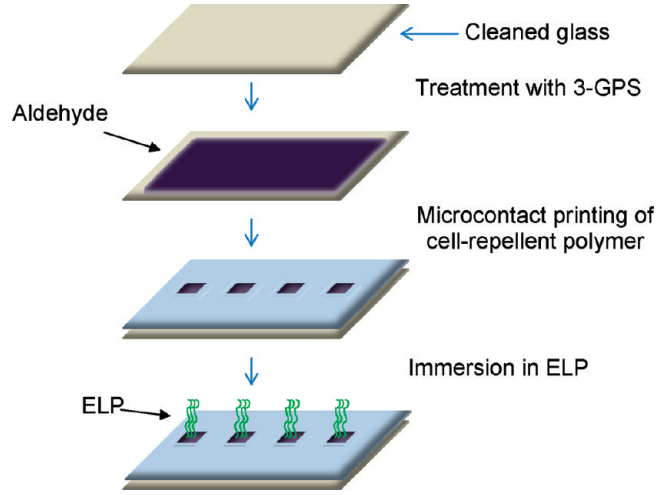
Preparation of ELP-Functionalized Glass Substrate. Cleaned glass plates (15 × 15 × 1 mm^3) were silanized by immersion in a 2% solution of 3-glycidoxypropyl trimethoxysilane (Sigma) in toluene for about 8 h. The silanized glass plates were rinsed with acetone and dried under a nitrogen stream. The plates were then reacted with 100 mM NaCl (pH 4.0) and 47 mM $NaIO_4$, and subsequently dried under a nitrogen stream.

The cell-repellent polymer was micropatterned using an oxidized polydimethylsiloxane (PDMS) stamp with micrometer-sized features. The masters for the elastomeric molds were fabricated from polished Si wafers by spin-coating the photoresist (AZ 1512, Clariant, Inc.) followed by processing with contact photolithography. The elastomeric stamps were fabricated by casting PDMS against the photoresist on a silicon master. The PDMS stamps were oxidized for 20 s using a commercially available hydrophilizer before microcontact printing. The stamp was inked with a 1% (w/v) solution of the cell-repellent polymer in a 50:50 (v/v) H_2O/ethanol mixture and brought into conformal contact with an aldehyde-derivatized glass surface, resulting in the transfer of the cell-repellent polymer to the regions of the surface that were in contact with the stamp. Subsequently, the micropatterned glass plate was incubated for 3 h with 1 mg/mL ELP in 50 mM sodium carbonate buffer (pH 9.2) and 5 mM $NaCNBH_3$. Unbound ELP was removed by washing with PBS (pH 7.4), and the plates were stored at 4 °C until use. The preparation procedure is illustrated in Scheme 1.

A cell-repellent polymer (MW = 30 000 Da, PDI = 2.3) based on polyethylene glycol methacrylate and methyl methacrylate (MMA) was synthesized by free-radical polymerization; details of the synthesis are described elsewhere (28). The micropatterns of the cell-repellent polymer on the surface were observed with optical microscopy and imaged using a CCD camera connected to a computer. The thickness of the micropatterns on the glass substrates was determined from a line profile of images acquired in air, using atomic force microscopy (Asylum Research, Santa Barbara, CA, USA) in contact mode with V-shaped silicon nitride cantilevers (Nanoprobe, Veeco, Plainview, NY; spring constant 0.12 N/m; tip radius 20−60 nm).

Preparation of ELP-Bacteria Conjugates. *Escherichia coli* K12 (Stratagene) and *Bacillus halodurans* (JCM 9153) were cultivated at 37 °C in LB (Luria−Bertani) medium. *Gluconacetobacter xylinus* (KCCM 40216) was cultivated at 26 °C in mannitol medium containing 25 g/L mannitol, 5 g/L yeast extract, and 3 g/L peptone. *Sphingomonas aromaticivorans* (KCTC 2888) and *Comamonas testosteroni* (DSM 6577) were cultivated at 30 °C in LB medium. The bacterial cultures were grown until the OD_{600} reached 1.0. After harvesting the bacterial cells by centrifugation, the supernatant was discarded and the pellet washed with PBS (pH 7.4). The bacterial cells were then treated for 15 min with 5 mM NHS (*N*-hydroxy succinimide) and 2 mM EDC (*N*-ethyl-*N*′-[3-dimethylaminopropyl] carbodiimide) in PBS (pH 7.4). Unreacted reagents were removed by centrifugation, and the bacterial pellets were resuspended and conjugated to 10 μM ELP in PBS (pH 7.4). The conjugation reaction was performed for 2 h at 25 °C, and the reaction was purified by three rounds of centrifugation.

Scheme 1. Schematic of the Procedure for Fabricating the ELP-Functionalized Glass Substrate



ELP-Mediated Bacterial Microarray. The ELP-functionalized glass substrate was placed in a polystyrene Petri dish on the cooling stage whose temperature could be controlled by circulating water in the range from 10 to 60 °C.

ELP-bacteria conjugates in LB medium (1 × 10^4 CFU/mL) was introduced to the ELP-functionalized glass substrate in LB medium, and the temperature was then increased to 37 °C (above the T_t). After 30 min, unbound bacterial cells were removed by washing with fresh LB medium. Surface-attached bacteria were characterized by phase-contrast microscopy (TS100-F, Nikon, Tokyo, Japan) and fluorescence microscopy (BX21, Olympus, Tokyo, Japan) at 37 °C, and field-emission scanning electron microscopy (FE-SEM). Bacterial detachment from the glass substrate was triggered by lowering the temperature to 28 °C (below the T_t).

Evaluation of the Viability of Surface-Attached ELP-*E. coli* Conjugates. The viable count procedure was performed to estimate the viability of surface-attached ELP-*E. coli* conjugates. ELP-mediated *E. coli* K12 microarrays were incubated in LB medium at 37 °C for 12 h. During the incubation, the temperature of each microarray was reduced to 28 °C at 2 h intervals, and the detached ELP-*E. coli* K12 conjugates were recovered. The collected ELP-*E. coli* K12 conjugates were then plated onto LB agar and the viable bacterial cells were determined by counting colony forming units (CFU).

RESULTS AND DISCUSSION

In the experiment, an ELP molecule was designed to have a T_t value in the range of 30−37 °C for optimal *E. coli* growth and activity. An ELP block copolymer was synthesized with an amino acid sequence of R_7W $[(VGVPV)_5]_{26}$ + $[(VGVPG)_{14}(KGVPG)]_8$, whose T_t was determined to be 33 °C (Figure 1). The ELP solution was transparent at temperatures

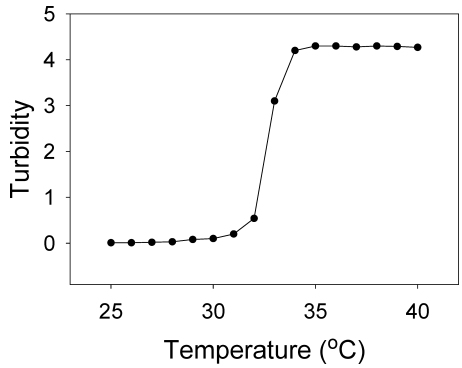


FIGURE 1. Phase transition of an ELP block copolymer with an amino acid sequence of R_7W $[(VGVPV)_5]_{26}$ + $[(VGVPG)_{14}(KGVPG)]_8$.

below 33 °C, but became turbid above 33 °C due to the formation of ELP aggregates. The phase transition was found to be reversible during repetitive temperature heating and cooling experiments.

Eight lysine residues were introduced into the ELP pentapeptide sequence for the fabrication of bacteria-ELP conjugates and an ELP-functionalized glass substrate. It was chosen for the conjugation of ELPs to NHS-derivative or aldehyde-derivative surfaces and for the appropriate T_t value in the range of bacterial incubation temperature. Generally, a lysine residue increases the T_t values of $[(VGVPG)_{14}$-$(KGVPG)]_8$ because of its hydrophilicity, but it was possible to adjust the T_t value of 33 °C by increasing the molecular weight of the peptide and fusing it to the sequence R_7W $[(VGVPV)_5]_{26}$.

PEG-branched cell-repellent polymer template was routinely fabricated by microcontact printing using a PDMS stamp. A 1% cell-repellent polymer solution in 50/50 (v/v) H_2O/ethanol was micropatterned onto the aldehyde-derivatized glass surface. Subsequently, ELP was introduced to the aldehyde-derivatized glass surface. The ELP-functionalized glass surface was prepared by forming a Schiff base between the amine groups of ELP and the aldehyde groups of the glass surface. Glass was chosen for its biocompatibility, nontoxicity, and ease of surface modification. The glass was modified to generate the aldehyde functional groups on its surface for the covalent binding of the lysine residues of ELP rather than adsorption of the peptide. An aldehyde-derivatized glass surface could offer the covalent binding of ELP molecules to the glass surface. Because the Schiff base formed between aldehyde and amine is chemically unstable and reversible, a reducing agent ($NaCNBH_3$) was applied for 3 h to form a stable secondary amine.

Figure 2A diagrams the ELP-functionalized glass with 10- and 20-μm negative features of cell-repellent polymeric template. Alexa Fluor 488-conjugated ELP ($T_t \approx 31$ °C) was used to verify the conjugation of ELP molecules onto the surface (Figure 2B) and the stable immobilization of ELP on the glass was confirmed by comparing the fluorescence intensity under harsh conditions, including a wide range of temperature (4−60 °C), pH (4−10), and ionic strength or salt concentration (0−4 M). Any significant reduction of fluorescence intensity was not observed under the conditions above (results not shown).

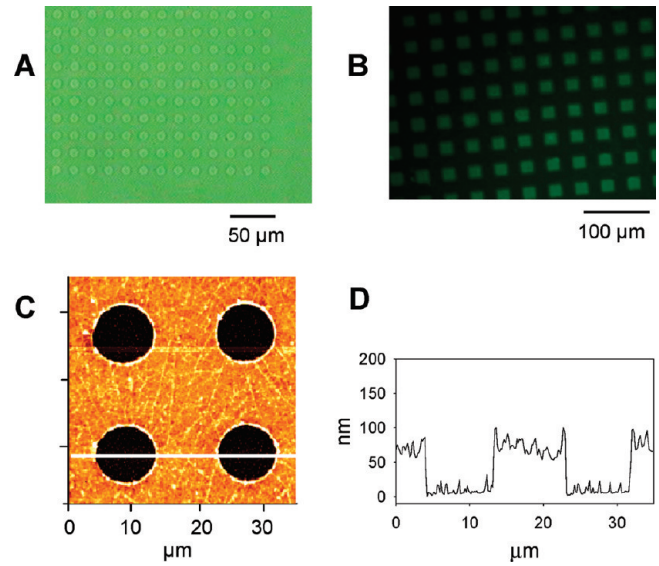


FIGURE 2. Cell-repellent polymer micropatterns on an ELP-functionalized glass substrate. (A) Optical image of 10 μm circular features of polymer and (B) fluorescence image of 20 μm square features of polymer with Alexa Fluor 488-conjugated ELP. (C) AFM image of 10 μm circular features of polymer. (D) Line profile of the AFM image in C.

The thickness of the cell-repellent polymeric template on the surface was measured using atomic force microscopy (AFM) (Figure 2C and D). The template thickness was about 60−70 nm, and was affected by the concentration of cell-repellent polymer and its surrounding hydrophobicity. Compared with the size of bacteria, the template thickness was negligible, but as shown in Figure 3C−F, the micropattern of the cell-repellent polymeric template prevented nonspecific bacterial adhesion. These results demonstrate that the cell-repellent polymeric template served as a chemical barrier against nonspecific bacterial adhesion through its PEG chains.

Bacteria were conjugated to ELP by the formation of an amide bond between the NHS-modified bacterial surface and the amine groups of ELP. The conjugation of ELP to the bacterial surface was confirmed by fluorescence detection using *E. coli* K12 tagged with Alexa Flour 488 (Figure 3A and B). We tried to get the surface concentration of ELPs on the bacterial surface. However, it was impossible to get the reliable quantitative fluorescence intensity due to the small size of bacterial cells. Instead of it, 10 μM of ELP was suggested to cover the bacterial cells by observing the reliable attachment and detachment of bacterial cells from the pretests. Well-localized *E. coli* microarrays on the ELP-functionalized glass surface that occurred at temperatures above the LCST of ELP can be seen in Figure 3C−F. Bacterial attachment was achieved through the specific binding of ELP-bacteria conjugates to ELP-functionalized glass through hydrophobic interactions between the ELP molecules.

The mechanism of bacterial attachment mediated by ELP molecules ($T_t \approx 33$ °C) was reversible. The surface-attached bacterial cells could be detached from the surface by decreasing the temperature below the LCST of ELP (Figure 4). The first detachment of a bacterial cell was shown at 32 °C,

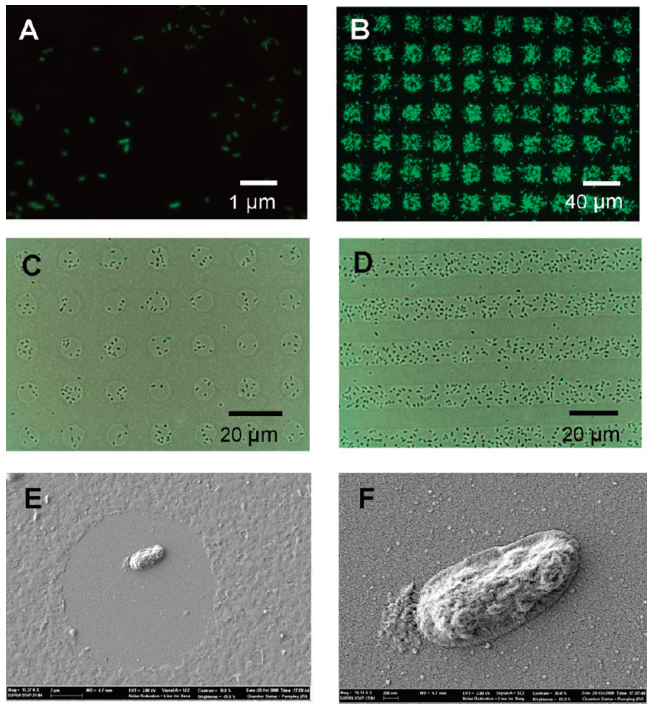


FIGURE 3. ELP-mediated *E. coli* microarray. Fluorescence images of (A) ELP (Alexa Fluor 488)-conjugated *E. coli* K12 and (B) microarray prepared using the *E. coli* shown in Figure 2A. Optical images of *E. coli* K12 microarrays with ELP micropatterns of (C) 10 μm circular features and (D) 10 μm stripe features. (E) FE-SEM image of the *E. coli* K12 microarray with an ELP micropattern of 10 μm circle features. (F) An enlarged image of E. 10-fold diluted ELP-bacteria conjugate in LB medium was used for the FE-SEM imaging of 10 μm circular features of microarray for imaging of FE-SEM. Fluorescence and optical images were taken at 37 °C.

and more than 90% of bacterial cells were detached from the surface at 28 °C by counting the cells remaining on the surface.

As the temperature decreased to below the LCST, the hydrophobic interactions between the ELP-*E. coli* K12 conjugates and the ELP-functionalized glass disappeared because of the phase transition to a hydrophilic state. Consequently, the bacterial cells detached from the glass substrate. Moreover, the reversible phase transition of the ELP molecules enabled the repetitive attachment of the newly prepared ELP-*E. coli* K12 conjugates to the ELP-functionalized glass surface (Figure 4C). A total of three cyclic experiments confirmed the reliable smart property of the ELP-mediated microarray. The nonspecific attachment of bacterial cells was less than 2% by counting cells attached on the outside of micropatterns each time.

The method described in this paper has advantages over previously described methods in terms of bacterial viability and activity. Rapid decreases in bacterial viability may cause performance problems in microfluidic systems, and the reliability of the systems cannot be guaranteed. In contrast, the ELP-mediated method can provide a feasible and simple way to solve these problems by removing the inactive cells through a simple change of temperature. When followed by the introduction of fresh ELP-bacteria conjugates to the microfluidic chamber and controlling the attachment, new bacterial microfluidic systems can be constructed. A viable

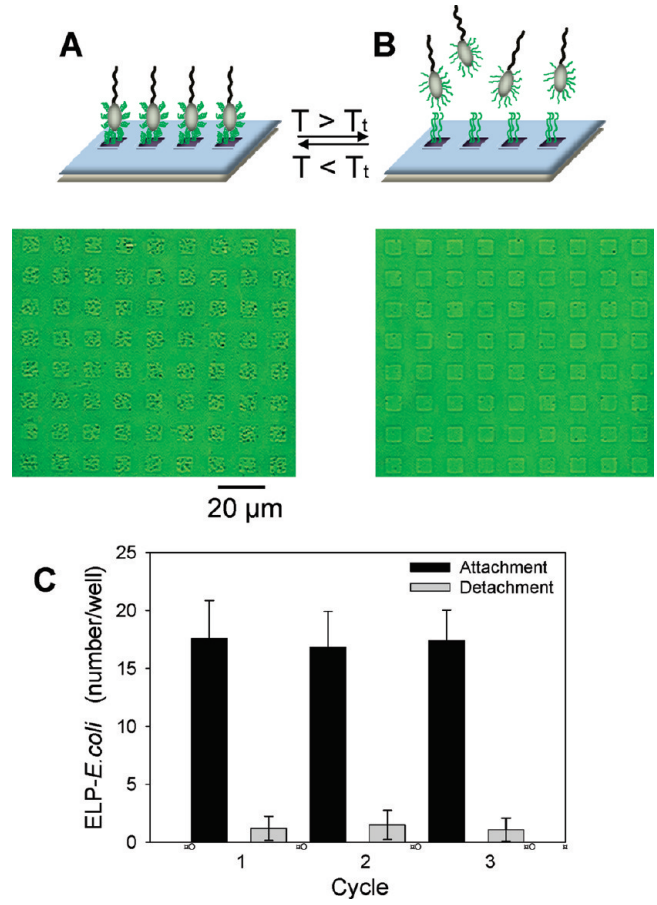


FIGURE 4. ELP-mediated controllable bacterial attachment and detachment. (A) At 37 °C above the LCST, ELP-*E. coli* K12 conjugates were attached to the ELP-functionalized glass surface through hydrophobic interactions. (B) At 28 °C below the LCST, the ELP-*E. coli* K12 conjugates were detached from the surface due to the phase transition of ELP molecules. (C) Three repetitive attachment (at 37 °C) and detachment (at 28 °C) of ELP-*E. coli* K12 conjugates.

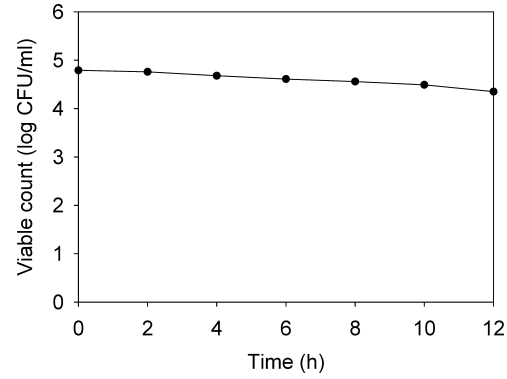


FIGURE 5. Viability of ELP-*E. coli* K12 conjugates detached from the surface.

count method was employed to estimate the viability of surface-attached ELP-*E. coli* K12 conjugates. As shown in Figure 5, the system showed consistent viability even during long-term immobilization.

The system was used to create microarrays of different types of bacterial species: *B. halodurans*, *G. xylinus*, *S. aromaticivorans*, and *C. testosteroni* (Figure 6). All individual bacterial microarrays showed reversible attachment and detachment controlled by a simple temperature change at

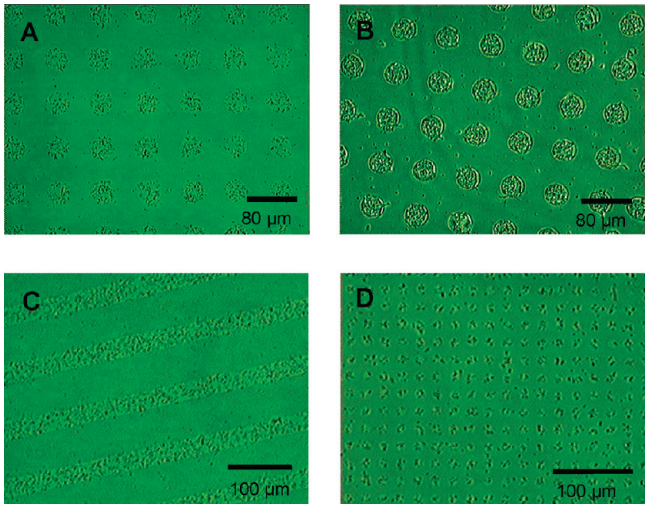


FIGURE 6. ELP-mediated bacterial microarrays. (A) *B. halodurans* microarray with an ELP micropattern of 40 μm circular features. (B) *G. xylinus* microarray with an ELP micropattern of 40 μm circular features. (C) *S. aromaticivorans* microarray with an ELP micropattern of 20 μm stripe features. (D) *C. testosteroni* microarray with an ELP micropattern of 10 μm circle features.

37 and 28 °C. These results suggest that it is possible to develop a microarray of multiple bacterial species attached to a single substrate using ELP molecules with different transition temperatures. In bacteria-based microfluidic and sensing systems, it is important to control bacterial attachment to avoid nonspecific bacterial adhesion. Moreover, for the consistent functionality of bacteria-based applications, it is also important to minimize or remove bacterial contaminants. We believe that this system fits these requirements and shows promise for multiple applications.

CONCLUSIONS

Controlled bacterial attachment and detachment was achieved using thermoresponsive ELP molecules. ELP was conjugated to both the bacterial surface and the glass substrate. Defined micropatterns of cell-repellent polymers were fabricated to prevent nonspecific bacterial adhesion. The attachment and detachment are switchable and can be controlled within an optimal temperature range that has no adverse effect on bacterial physiology. In addition, the conjugation of ELP to bacteria has little effect on bacterial viability. This ELP-mediated approach can be considered a generic method for bacterial micropatterning, and this method can be applied to bacteria-based microfluidic applications and sensing platforms.

Acknowledgment. This work was supported by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2008-0217-D01314) and a grant from the Korea Institute of Machinery and Materials.

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AM9002364