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Direct-Write Patterning of Bacterial Cells by Dip-Pen Nanolithography

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Supporting Information

ABSTRACT: The ability of dip-pen nanolithography (DPN) to generate nano- or microarrays of soft or hard materials (e.g., small molecules, DNA, proteins, nanoparticles, sols, and polymers) in a direct-write manner has been widely demonstrated. The transporting of large-sized ink materials such as bacteria, however, remains a significant challenge with this technique. The size limitation of the water meniscus formed between the DPN tip and the solid surface becomes a bottleneck in such diffusion-based molecular transport experiments. Herein, we report a straightforward "stamp-on" DPN method that uses a nanostructured poly(2-methyl-2-oxazoline) hydrogel-coated tip and carrier agents to generate patterns of micrometer-sized *Escherichia coli* JM 109 bacterial cells. We demonstrate that this approach enables the deposition of a single bacterial cell array on a solid surface or arrays of layers of multiple cells by modulating the viscosity of the "ink" solution. Fluorescence microscopy images indicated that the deposited bacterial cells were kept alive on Luria−Bertani-agar layered solid surfaces after DPN patterning.

The immobilization of microorganisms, such as bacterial cells, onto solid substrates has received increased attention for use in cellular biology, drug delivery, biofilms, biosensors, torque-generators, and biomolecular motors.[1−6] Microcontact printing, replication molding, and photo- or electron-beam lithography are common indirect methods employed to fabricate affinity arrays consisting of small molecules or prealigned templates that can subsequently direct the attachment of bacterial cells (deposited from solution or by stamping).[7−9] For example, Mirkin and co-workers have fabricated microarrays of motile bacterial cells onto predesigned 16-mercaptohexadecanoic acid patterned microarrays and prealigned holed microarrays.[6,9] Such indirect approaches, however, involve complicated stepwise procedures that have a fundamental limitation with respect to the multiple patterning ability and number of materials that can be treated within a microscopic field of view.[1]

In contrast to the aforementioned patterning methods, dip-pen nanolithography (DPN) is a direct-write scanning probe lithographic method.[10,11] DPN uses an "ink"-coated atomic force microscope (AFM) tip to deliver ink materials to a surface through the water meniscus formed between the tip and the substrate surface. The use of DPN for the generation of arrays of a variety of materials such as organic thiols,[10] DNA,[12] peptides,[13] nanoparticles,[14] collagen,[15] polymers,[16] and proteins[17] have been demonstrated. Furthermore, the Salaita group has recently used DPN to prepare patterns of polyelectrolytes for the control of ligand spatial organization in membranes and cells.[18] In addition, because of its unique direct-write capability, DPN can be used to deposit multiple compounds, sequentially or in parallel, precisely and exclusively to specific locations.[1] Although DPN is highly versatile and applicable to biomolecular patterning at nanometer resolution, the transport and deposition of large-sized ink materials through the water meniscus remains a significant challenge with this technique.[19] Many strategies have been employed to overcome this challenge by making use of alternate tips such as spore cells-terminated tips,[20] polymer pen, stamp tip, and fountain tip.[21] To the best of our knowledge, however, no studies have reported the successful patterning of large bacterial cells using a direct-write DPN technique.

We report here on the deposition of *Escherichia coli* (*E. coli*) JM 109 bacterial cells onto chemically modified solid substrates by DPN. We also demonstrate that our approach enables control of the number of bacterial cells transported from the tip to the solid surface.

Central to the deposition of the bacterial cells to solid surfaces were the use of a specialized AFM tip as well as the ink carrier materials. The former component is a nanostructured poly(2-methyl-2-oxazoline) (PMeOx)-coated AFM tip recently developed by our group for the preparation of arrays of large adeno-associated viruses.[19] In our previous work, we showed that diffusion of absorbed virus particles (ca. 25 nm) from the tip was enhanced by the biomolecule-repellent "Stealth"[22]

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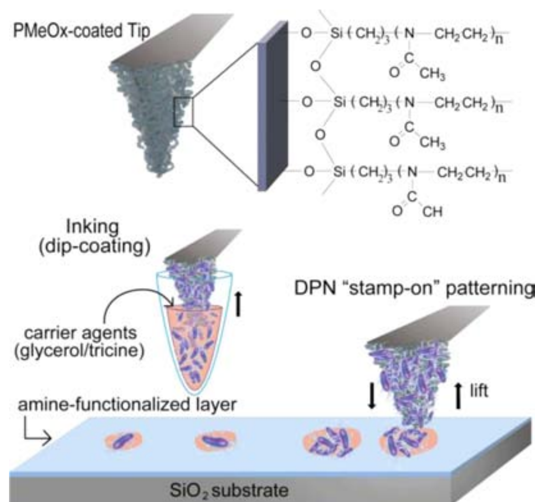
behavior of the PMeOx-coated tip surface. However, although the use of the PMeOx-coated polymer tip is suitable for the delivery of large biomaterials, it is not capable of transporting the approximately 1−2 μm or larger in length *E. coli* bacterial cells by diffusion through the submicrometer-sized water meniscus (Supporting Information [SI], Figure S1). With regards to the latter component, the use of glycerol and tricine as carrier inks offers two advantages in the direct deposition of bacterial cells. First, it protects *E. coli* bacterial cells from drying and denaturing while on the tip.[23] Second, the increased viscosity and interaction, known as an "accelerator" effect,[23] of the ink solution facilitates cell delivery during the patterning process. When the tip makes contact with the surface, the coated ink solution is transferred onto the amine-functionalized surface from the "sponge-like" hydrogel tip by the "stamp-on" approach of DPN (Scheme 1).

Scheme 1. Schematic Representation of "Stamp-on" DPN of Bacterial Cells on Modified Surfaces



In a typical experiment, we chemically modified the surface of a silicon oxide AFM tip (M2N, Inc., Korea, spring constant = 40 N/m, model = STP4, tip-end size = 880 nm) with 11-iodo-undecyltrichlorosilane. This I-functionalized layer was then polymerized by ring-opening polymerization of 2-methyl-2-oxazoline monomers, resulting in the formation of a nano-structured PMeOx-hydrogel with a tip-end size of ∼1100 nm (Scheme 1 and Figure 1). All "stamp-on" DPN patterning was done under ambient conditions at 30−35% relative humidity with a contact time of 1 s (contact force = 2000 nN) using a XE-100 AFM system (Park systems, Inc., Korea). Fluorescence images were taken 2 h after patterning with a confocal laser scanning microscope system (LSM 510, Zeiss). *E. coli* JM 109 strain was cultured in Luria−Bertani (LB) broth at 37 °C for 12 h with vigorous shaking. The Live/Dead Bac-Light Bacterial Viability Kit (L-13152, Molecular Probes) was used to label bacterial cells. Living bacteria fluoresce green (SYTO9) and dead bacteria fluoresce red (propidium iodide) (SI, Figure S1).

For proof-of-concept, two specialized *E. coli* bacteria inks were prepared to allow transport of the micrometer-sized cells from the tip to the solid surfaces of interest. One ink solution contained 10% glycerol and 100 mM tricine as carrier agents, and the other included 20% glycerol with 100 mM tricine. The viscosity of the ink, which would be expected to control the number of cells transported during the patterning process, was

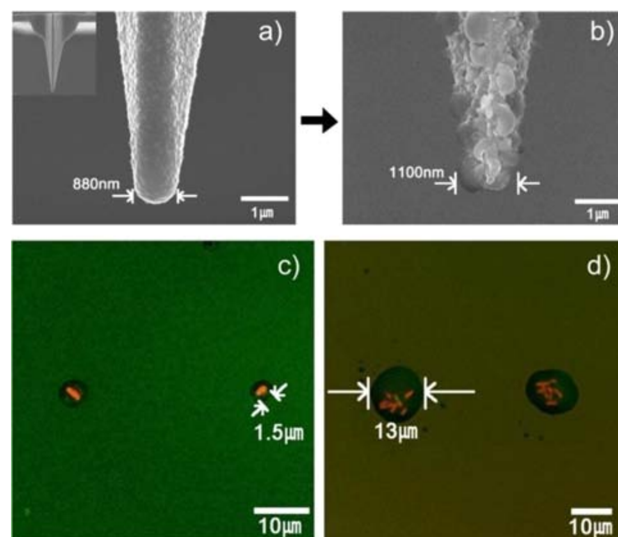


Figure 1. (a) Typical SEM image of a bare silicone oxide AFM tip. (Inset: SEM image of an entire bare tip.) (b) SEM image of a nanostructured PMeOx-coated tip (c) Fluorescence microscopy image of a pattern of individual *E. coli* JM 109 bacterial single cell. (c) Fluorescence microscopy image of *E. coli* bacterial cell (7−8 cells per one "stamp-on" feature) patterns.

modulated by varying the glycerol concentration (Scheme 1). As shown in Figure 1c, we successfully generated patterns of single *E. coli* JM 109 bacterial cells on amine-functionalized silicon oxide surfaces with a 10% glycerol ink solution. The adhesion of *E. coli* cells to the surface likely occurs via an electrostatic interaction between negatively charged lipopoly-saccharide groups on the surface of the bacterial cells and the positively charged amine-functionalized surface of the sub-strate.[6] By increasing the glycerol concentration to 20%, we were able to generate 13-μm-sized dot features comprising 7−8 cells (Figure 1d). In addition, 25−30 cells could be deposited from a 60% glycerol ink solution (SI, Figure S2a). These results indicate that the number of bacterial cells deposited on the surface can be controlled by simply varying the viscosity of the ink solution (SI, Figure S2b). Unlike conventional DPN, which allows the size of the pattern to be adjusted by changing humidity and tip contact time,[10] the number of cells deposited using our methodology was almost independent of such parameters. Such findings are consistent with the results of studies performed with large-sized ink materials using a polymer tip.[24] However, it seems that the number of bacterial cells coated onto the tip surface is limited by the size of the cells as only nine features comprising 1−2 bacterial cells could be deposited (SI, Figure S2c).

To validate the methodology, patterning was repeated using bare and amine-functionalized tips. No bacterial cell patterns were observed in either case (SI, Figure S3), indicating that the PMeOx-hydrogel tip is effective at the required transport and deposition. The carrier agents that were included in the ink solution to increase the mobility of the bacterial cells played a key role in the patterning process. Inks without carrier agents could not deposit bacterial cells with the PMeOx-coated tip. Transport seems to be dominated primarily by diffusion of viscous ink from the PMeOx-coated tip to the surface. Note that the bacterial cells in the fluorescence microscopy images fluoresce red (propidium iodide), showing that the cells die upon patterning. Cell death was most likely attributed to

damage of the cell membrane that occurred during the drying step of the patterning process (live bacteria with intact membranes fluoresce green (SYTO9)) (SI, Figure S1).[6,9] Fluorescence micrographs taken 40 min after the bacteria were coated to the tip confirm that it is due to denaturing on the surface rather than DPN itself that causes cell death (SI, Figure S4). While coated to the tip, the cells fluoresce green and thus are alive at this stage of the patterning process.

The efficient and simple patterning of living bacterial cells on surfaces is potentially significant to a broad range of fields including cell engineering, biomotors, cell growing, and cell−cell or −protein interactions in cellular resolution on a solid surface. We prepared LB agar-coated solid substrates by placing the amine-functionalized solid substrate in 0.2 wt % agar solution for 10 min (Figure 2a). LB agar is commonly used for growing cells.[25] As shown in fluorescence microscopy images (Figure 2b), the patterned bacterial cell arrays fluoresce green, indicating that the deposited bacteria are living. AFM topography shows that each dot consists of 1−2 bacterial cells with a 1−2.5 μm in length (Figure 2c). To verify whether the contact pressure causes cell death, we deposited bacterial cells on LB agar-coated silicon oxide surfaces with different contact forces (100, 4000, and 10000 nN) from a 20% glycerol ink solution (SI, Figure S5). All of the bacterial cells fluoresce green, indicating they remain alive even following deposition using a high contact force of 10000 nN.

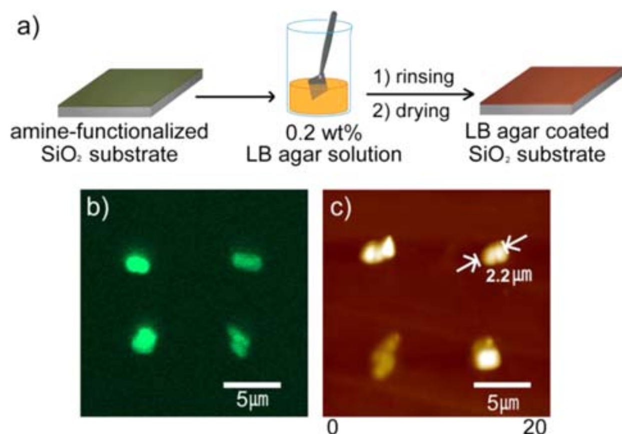


Figure 2. (a) Schematic illustration of the process for fabricating Luria−Bertani (LB) agar-coated silicone oxide substrate. (b) Fluorescence microscopy image of an *E. coli* JM 109 bacterial cells pattern. (c) AFM topographic image of bacterial cell array.

In conclusion, we have developed a straightforward "stamp-on" DPN method that uses a nanostructured PMeOx-coated tip and carrier agents to generate patterns of micrometer-sized *E. coli* JM 109 bacterial cells. To the best of our knowledge, this is the first demonstration of DPN patterning of such large bacterial cells in a direct-write manner. We have shown that this approach enables the deposition of a single bacterial cell on a solid surface as well as a layering of multiple cells through a modulation of carrier solution viscosity. Furthermore, we also showed that a living bacterial cells array was successfully generated on an LB agar-coated solid surface.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional characterization, including atomic force microscopic images and fluorescence microscopy images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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